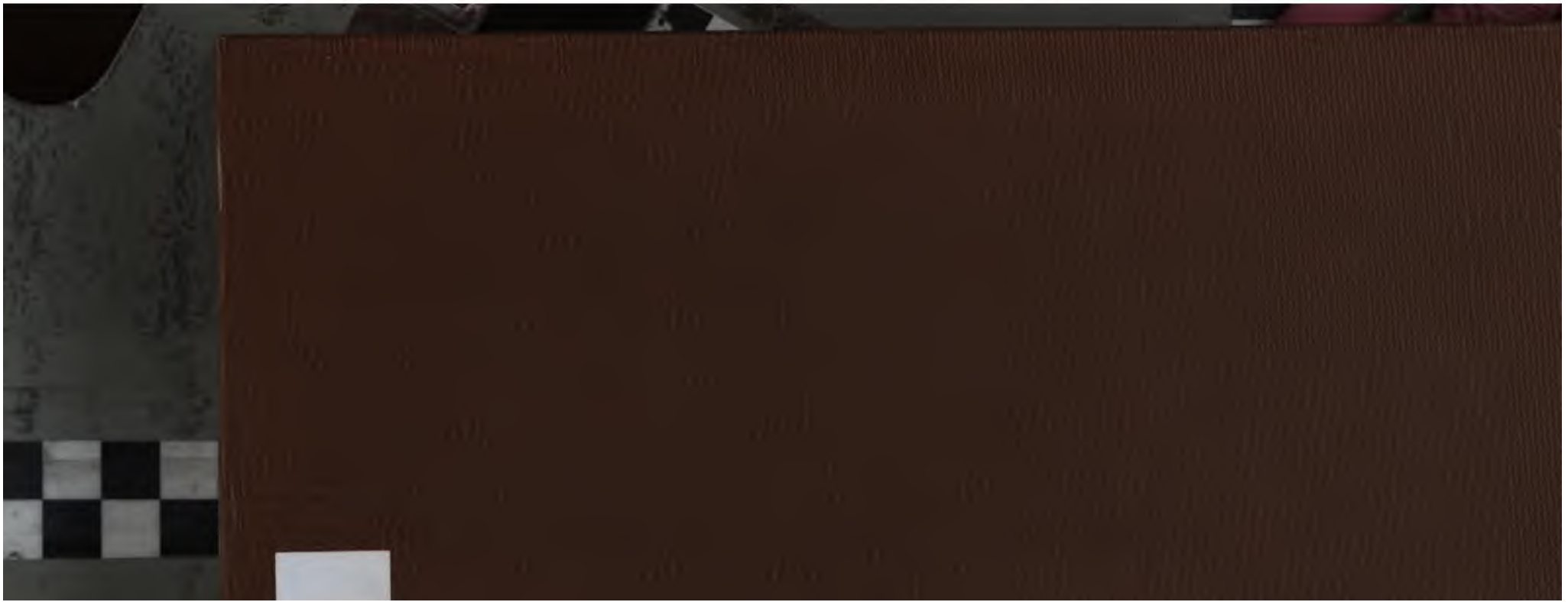

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OF

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FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

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… **systemic syphilis** Dr. Loyd Thompson recommen… Mercurialized Serum **intravenously.**

(Journal American Medical Association, Dec. 19, 1914, p. 2182; May 1, 1915, p. 1471; Mulford Digest, May, 1915.)

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CONTRIBUTIONS TO THE BIOCHEMISTRY OF IODINE.

II. THE DISTRIBUTION OF IODINE IN PLANT AND ANIMAL TISSUES.

PART II.

By A. T. CAMERON.

(From the Department of Physiology and Physiological Chemistry, University of Manitoba.)

(Received for publication, July 6, 1915.)

In Part I of this paper[1] I gave a summary of known data, together with a number of fresh facts, especially from results obtained for marine species. A number of conclusions were drawn, of which the following may be regarded as most important.

"Iodine appears to be an invariable constituent of all marine Algae, in amounts greater than 0.001 per cent. There does not appear to be any specific difference between the amounts present in Brown and Red Algae. . . . . The results for Green Algae are too few to permit of a similar generalisation. Distinct variation of iodine content can occur in the same species, growing under almost the same conditions, and in different but closely related species. . . . On the other hand, the marked difference between fresh-water plants and vegetables on the one hand, and marine Algae on the other, is due to difference in the iodine content of the environ-

[1] Cameron, A. T., *Jour. Biol. Chem.*, 1914, xviii, 335. The following errata are in Part I:

p. 350, line 43, should read "Fucus evanescens, Nanaimo, B. C. * * * * 0.013 per cent."

p. 364, line 7, should read "Squalus sucklii (pups) * * * * 0.00 per cent."

p. 367, ref. 85, should read "*Arch. Int. Med.*, iv, p. 261, 1909."

p. 368, line 2, should read "Dog-fish (Squalus sucklii) (f); 0.195 (per cent iodine); * * * * 0.011 (mg. iodine per kg. animal)."

Further, Dr. A. Hunter has drawn my attention to the figure quoted as maximum iodine content for sheep's thyroid (p. 367), which should be "sheep thyroids, 0.58 per cent" (Simpson, S., and Hunter, A., *Quart. Jour. Exper. Physiol.*, 1911, iv, 263).

ment, and therefore in the diet of the plants. The difference in content in different species of vegetables (Bourcet) parallels that in different species of Algae growing under similar conditions, and suggests a specific quantitative action of the plant cell in retaining iodine.

"Iodine is present in appreciable quantities in certain tissues of all marine species. As we get higher in the scale there is more differentiation (and probably less total iodine in the whole organism) until in vertebrates thyroid tissue alone is of consequence. . . .

"Of mammalian tissue, the thyroid alone is of importance in connection with the storage of iodine. . . . It (the amount of iodine) is less than 0.001 per cent in all non-thyroid tissue.

"In thyroid tissue marked variations of iodine content occur, both in individuals of the same species, and in different species. Such variations are all traceable to differences of diet.

"Iodine is an invariable constituent of normal thyroid tissue, and under normal conditions the diet always contains sufficient iodine for the upkeep of a minimal amount. The minimal quantity appears to be of the order 0.01 per cent, the maximal quantity so far observed being 1.16 per cent.

"Three different tissues of marine animals, reported on in this paper, contain iodine in marked quantity, the test of the tunicate *Pyura*, the outer cuticle of the horse-clam *Schizothoerus*, and the inner tube of the worm *Diopatra*."

Of the papers dealing with iodine which have appeared during the past year the following bear directly or indirectly on the distribution of the element in living tissues.

Seidell and Fenger have published further data concerned with the seasonal variation in the iodine content of the thyroid[2] and consider that their results indicate that temperature change is the greatest factor in the cause of the seasonal variation. They state: "On the basis of our present knowledge it therefore seems improper to lay much emphasis upon the factor of diet as an explanation of the seasonal variations in thyroid activity." Their evidence will be discussed later.

Hunter and Simpson[3] find that dry thyroid tissue of sheep from the Orkneys, which feed normally during the winter to a very large extent on sea-weed, contains quantities of iodine varying from 0.418 to 1.050 per cent (different individuals). They are of the opinion that this result confirms the general correctness of the view expressed in Part I of this paper, "that the variations in the iodine content of thyroid tissue are all referable to differences of diet."

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[2] Seidell, A., and Fenger, F., *Bull. Hyg. Lab., U. S. P. H. and M.-H. S.*, 1914, xcvi, 67.

[3] Hunter and Simpson, *Jour. Biol. Chem.*, 1915, xx, 119.

Fenger[4] finds that human fetal thyroids contain iodine. The amounts are somewhat small in comparison with those in other animals. The result confirms my conjecture that the negative figures for new-born children's thyroids previously recorded were due simply to the less accurate analytical methods available.[5]

The investigators named have all employed Hunter's method of analysis. Blum and Grützner have published a series of papers of which I have been unable to consult the originals.[6] These papers deal chiefly with the condition of the iodine in the thyroid and other tissues. Kendall[7] has presented definite data to show that more than one iodine compound is present in the thyroid, and that these different compounds produce different physiological effects, which are not producible by inorganic iodides.

In a further visit to the Pacific Coast Station of the Dominion Biological Board (at Departure Bay, B. C.) during the summer of 1914, I was enabled to obtain a considerable amount of fresh material which has since been analyzed in this laboratory. The results of the analyses are embodied in this paper.

The material was collected with a view to throwing further light upon (1) the distribution of iodine in Algae, (2) the presence of iodine in such tissues as the ascidian test, the annelid worm-tube, and the dermis of the foot of the horse-clam, and (3) the degree of variation of iodine in fish thyroids. In addition certain other thyroid material was obtained.

The material was almost invariably either preserved in absolute alcohol, or, if of a suitable nature, air-dried. In some cases, when a supply of alcohol was not available, the material was preserved in formaldehyde. Some evidence will be quoted to show that this latter method results in loss of iodine. Before analysis the material was evaporated and dried at 100°C. to constant weight. In these analyses I have discarded Hunter's method, and have used Kendall's method as revised by himself.[8] This I have found to be very satisfactory. In my hands it appears to have two advantages over Hunter's method: (1) the greater certainty of a negative result in the absence of iodine, and (2) the greater

[4] Fenger, F., *Jour. Biol. Chem.*, 1915, xx, 695.

[5] Cameron, *ibid.*, 1914, xvi, 466.

[6] Blum, F., and Grützner, R., *Ztschr. f. physiol. Chem.*, 1914, xci, 400, 450; 1914, xcii, 360; through *Chem. Abstr.*, 1914, viii, 3587, 3588; 1915, ix, 649.

[7] Kendall, E. C., *Jour. Biol. Chem.*, 1915, xx, 501.

[8] Kendall, *ibid.*, 1914, xix, 251.

convenience of bromine as an oxidizing agent, instead of hypochlorite. It is perhaps a little less accurate, since I find that duplicate analyses do not yield such good agreement. Numerous tests with organic materials containing no iodine, and with known quantities of iodide have satisfied me that I have employed the method correctly. With regard to the degree of accuracy Kendall states:[9] "The results of over two thousand determinations of iodine show that the method will detect as little as 0.005 mgm. of iodine. One great advantage of the method is the entire absence of any test for iodine in cases where there is no iodine present. Where iodine ranges from 1 to 5 mgm. duplicate determinations should not differ more than 0.01 to 0.02 mgm., which means a difference in burette readings of but 0.1 to 0.2 cubic centimeter."

Working with 0.5 gram of material, this gives a minimal limit of 0.001 per cent, and a possible error of 2 per cent in duplicate analyses. With small amounts of iodine the difference is occasionally much greater, and the cause of error is traceable, as Kendall indicates, to over-acidification with phosphoric acid after the initial fusion.

In doubtful cases I have carried out duplicate analyses where sufficient material was available. In one or two cases this could not be done, and the results must for the present remain unchecked; this illustrates the disadvantage, at present irremediable, of carrying out the analyses at a place so distant from the source of the material.

In many cases, also, the material (*e.g.*, certain tunicates, and annelid worms) is scarce, and when obtained by dredging, the amount obtained is more or less a matter of chance, so that it is often difficult to obtain quantities even large enough for a single analysis.

The Iodine Content of Sea Water.

An attempt to confirm Gautier's results for sea water (compare Part I, p. 342) and to trace a parallelism between iodine content and specific gravity of the water has not met with success. Several samples of the water were evaporated, following Gautier's procedure, to the point of crystallization, after addition of a little potassium carbonate and hydroxide, and were brought here in

[9] Kendall, *Jour. Biol. Chem.*, 1914, xix, 256.

that condition, but subsequent analysis (after a somewhat long period) following Gautier's directions, gave negative results both for organic and inorganic iodine, indicating either a misuse of the method, a flaw in the method, or some cause of error connected with the delay between the initial and final stages of the analyses. I hope to be able subsequently to examine this problem more exactly and completely at the Pacific Coast Station. In the meantime I shall assume that the iodine content of sea water is parallel to the specific gravity.

Dr. McLean Fraser and I have carried out a series of observations, during the four months June to September inclusive of 1914, of the specific gravity of the surface water at various parts of the British Columbia Coast.[10] Our results lead to the conclusion that the relatively closed area (Area i) between Vancouver Island and the mainland, limited to the north by the Seymour Narrows, and the Yucultas, and to the south by the passages between the islands lying southeast of Gabriola Island, and forming the southern limit of the Strait of Georgia, possesses a somewhat lower average density (1.018 to 1.019) than waters further to the north or to the south (Area ii; average density, 1.021 to 1.022). We have found that this appears to condition the distribution of the Alga *Macrocystis*, and the shellfish *Haliotus* (absent from Area i), and also that the total halogen content of the waters can be regarded as proportional to the specific gravity. I have also obtained definite evidence that the growth of kelp is largely dependent on the salinity of the containing waters.[11] The material of which the analyses follow was largely collected within Area i. It seems probable that systematic comparison of the same species in the two areas would show a definite difference in iodine content. For comparison in future work the exact localities are given; material from different points within Area i should be capable of direct comparison.

Area i.

(a) At the Biological Station, Departure Bay, or at points within half a mile of it.

[10] Fraser, C. M., and Cameron, A. T., Variations in Density and Temperature in the Coastal Waters of British Columbia, *Contributions to Canadian Biol.*, Ottawa (in press).

[11] Cameron, The Commercial Value of the Kelp-Beds of the Pacific Coast of Canada, *ibid.* (in press).

(b) North-west of the Station, in the neighborhood of Hammond Bay and the "Lagoon."

(c) Near Snake Island, two miles east of the Station.

(d) From the sand flats off Protection Island, two miles south-east of the Station.

(e) In False Narrows, about eight miles south-east of the Station.

(f) North of Breakwater Island, two miles east of False Narrows.

(h) At Nanoose, ten miles north-east of the Station.

(i) At North West Bay, twenty miles north-east of the Station.

(j) At Belle Chain, fifty miles south-east of the Station.

(k) At Trail Bay, on the B. C. mainland.

Area ii.

(g) South of Mudge Island, two miles south of False Narrows.

(l) East of Ruxton Island, fifteen miles south-east of the Station.

(m) West of Porlier Pass, twenty miles south-east of the Station.

(n) Near the mouth of Barkley Sound, West Coast of Vancouver Island.

(o) Off Haddington Island, north of Vancouver Island.

(p) Off Suquash, north of Vancouver Island.

(q) Off Rose Spit, Graham Island (open ocean and high salinity).

Material dredged was obtained between 5 and 15 fathoms. Fraser and Cameron have shown in the communication already referred to that water from such depths shows a slightly higher density than that at the surface.

It may be mentioned here that I was informed by different workers at the Biological Station that the sand flats between Protection and Newcastle Islands (d), which are exposed at moderately low tides, smell distinctly of iodoform. I confirmed this personally, and found further that after collecting material (chiefly worm-tubes) with my hands from the sand of these flats during one or two hours the hands also smelled distinctly of iodoform. It will be observed that most of the material obtained from these flats is rich in iodine. Other sand flats, apparently similar and similarly rich in iodine-containing material, did not show this phenomenon.

Iodine Content of Plants.

The only plants examined were Algae. A large number of the rarer species were named for me by Mr. A. Klugh, to whom my thanks are due. In a number of cases numerous samples of the same species were obtained in the same place for purpose of comparison, and the results of these are dealt with in special tables.

Sub-class.	Family.	Species.	Sample No.	Date obtained.	No. of plants.	Where obtained.	Amount taken.	Iodine found.	Iodine.
							gm.	*gm.*	*per cent*
Diatomaceae		Melosira (Sp. ?)	9	5-8/v	Numerous	(a); filtered from surface water	0.250	None	0.00
							0.035	None	0.00
Chlorophyceae	Ulvaceae	Monostroma fuscum	10	20/viii	"	(a); between tides	0.500	0.000032	0.006
			11	"	"	"	0.500	0.000011	0.002
			12	"	"	"	0.470	0.000071	0.015
			13	"	"	"	0.500	0.000147	0.029
		Ulva lactuca rigida	14	6/vi	Several	(h); low tide	0.500	0.000023	0.005
			15	19/vi	"	(l); dredged	0.500	0.000055	0.011
		Enteromorpha linza	16	9/vi	Numerous	(e); low tide	0.161	0.000057	0.035
			17	26/vi	"	(m); at surface	0.427	0.000025	0.006
			18	20/viii	"	(a); between tides	0.083	Trace	Present.
	Cladophoraceae	Cladophora stimpsoni	19	27/v		(a)	0.500	0.000047	0.009
	Codiaceae	Codium fragile californicum	20	20/viii	Several	(a); between tides	0.144	0.000005	0.003
Phaeophyceae	Encoliaceae	Scytosiphon lomentarius	21	6/vi	Numerous	(h); low tide	0.500	0.000071	0.014
	Desmarestiaceae	Desmarestia viridis	22	8/vi	"	(e); " "	0.386	0.000103	0.026

Sub-class.	Family.	Species.	Sample No.	Date obtained.	No. of plants.	Where obtained.	Amount taken.	Iodine found.	Iodine.
							gm.	*gm.*	*per cent*
Phaeophyceae									
	Desmarestiaceae								
		Desmarestia ligulata	23	20/viii	Numerous	(a); between tides	0.500	0.000021	0.004
		Desmarestia ligulata herbacea	24	19/vi	A few	(i); dredged	0.500	0.000288	0.058
	Chordariaceae								
		Leathesia diformis	25	13/v	Numerous	(a); between tides	0.500	0.000070	0.014
			26	20/viii	"	(a); "	0.351	0.000033	0.009
			27	"	"	(a); "	0.556	0.000062	0.011
		Chordaria flagelliformis	29	8/vi	"	(c); low tide	0.443	0.000103	0.023
	Laminariaceae								
		Laminaria bullata (young plants)	30	10/vi	Several	(a); below low tide	0.432	0.001164	0.270
		Laminaria bullata (old plants)	31	"	"	"	0.500	0.000863	0.173
							0.500	0.000886	0.177
								(Mean	0.175)
		Laminaria saccharina (young plants)	32	"	"	"	0.405	0.000834	0.206
		Laminaria saccharina (old plants)	33	"	"	"	0.500	0.000394	0.079
							0.500	0.000383	0.077
								(Mean	0.078)
		Costaria turneri	34	10/vi	Several	(a); between tides	0.500	0.000143	0.029

		Nereocystis lütkeana								
		I	frond	35	26/v	1	(a)	0.260	0.000708	0.272
			float	36	"			0.0266	0.000053	0.20
			stipe	37				0.0518	0.000158	0.305
		II	frond	38	"	"	"	0.296	0.000737	0.249
			float	39				0.0433	0.000083	0.19
			stipe	40				0.0723	0.000190	0.263
		III	frond	41	26/v	1	(a)	0.494	0.001272	0.257
			float	42				0.132	0.000113	0.086
			stipe	43				0.133	0.000406	0.305
		IV	frond	45	"	"	"	0.494	0.001353	0.274
			float	46				0.113	0.000126	0.111
			stipe	47				0.128	0.000269	0.210
		V	frond	48	"	"	"	0.500	0.000870	0.174
			float	49				0.452	0.000725	0.145
			stipe	50				0.388	0.001045	0.269
		VII	frond	56	"	"	"	0.500	0.001082	0.216
			float	57				0.500	0.001311	0.262
			stipe	58				0.500	0.001376	0.275
		VIII	frond	60	27/v	"	"	0.500	0.001441	0.288
			float	61				0.500	0.000979	0.196
			stipe	62				0.500	0.001257	0.251
		IX	frond	63	"	"	"	0.500	0.000567	0.113
			float	64				0.500	0.000292	0.058
		X	frond	65	10/vi	Several	"	0.500	0.001252	0.250
			float	66				0.425	0.000554	0.130
			stipe	67				0.500	0.000666	0.133
								0.281	0.000374	0.133
									(Mean	0.133)
			holdfast	68				0.500	0.000734	0.147

Sub-class.	Family.	Species.	Sample No.	Date obtained.	No. of plants.	Where obtained.	Amount taken.	Iodine found.	Iodine.
							gm.	*gm.*	*per cent*
Phaeophyceae									
	Laminariaceae								
		Nereocystis lütkeana							
		XI frond	69	8/vii	Several	(j)	0.500	0.000489	0.098
		XI float and stipe	70				0.500	0.000972	0.194
		XII frond	71	25/vii	"	(o)	0.500	0.000391	0.078
							0.462	0.000375	0.081
								(Mean	0.079)
		XII float	72				0.500	0.000834	0.167
							0.217	0.000362	0 167
								(Mean	0.167)
		XII stipe	73				0.500	0.000328	0.066
							0.533	0.000347	0.065
								(Mean	0.065)
		Macrocystis pyrifera frond	74	23/vii	"	(p)	0.500	0.001053	0.210
							0.500	0.000954	0.191
								(Mean	0.200)
		Macrocystis pyrifera float	75				0.500	0.000818	0.164
		Macrocystis pyrifera stipe	76				0.500	0.000873	0.175
							0.500	0.000827	0.165
								(Mean	0.170)
		Macrocystis pyrifera holdfast	77				0.500	0.001170	0.234
		Alaria tenuifolia	78	8/vi	1	(e); low tide	0.500	0.000137	0.027
	Fucaceae								
		Fucus furcatus (? inflatus)	79	10/vi	Several	(a); between tides	0.500	0.000214	0.043
							0.500	0.000211	0.042

			80	20/viii	"	"	0.500	0.000086	0.017
			81	"	"	"	0.500	0.000077	0.015
			82	"	"	"	0.500	0.000126	0.025
							0.500	0.000152	0.030
								(Mean	0.027)
			83	20/viii	Several	(a); between tides	0.500	0.000056	0.011
		Fucus evanescens	84	10/vi	"	"	0.500	0.000144	0.029
							0.500	0.000141	0.028
								(Mean	0.028)
			85	20/viii	"	"	0.500	0.000078	0.016
			86	"	"	"	0.500	0.000096	0.019
			87	"	"	"	0.500	0.000071	0.014
			88	"	"	"	0.500	0.000090	0.018
							0.500	0.000073	0.014
								(Mean	0.016)
			89	"	"	"	0.500	0.000057	0.011
Rhodophyceae	Gelidiaceae								
		Chondrus crispus	90	"	"	"	0.500	0.000045	0.009
	Rhodophyllidaceae								
		Euthora fruticulosa	91	10/vi	Numerous	(a); very low tide	0.500	0.000265	0.053
	Rhodomelaceae								
		Polysiphonia tenuistriata	92	8/vi	"	(e); low tide	0.500	0.000046	0.009
		Polysiphonia tubulata	93	20/viii	"	(a); very low tide	0.458	0.000032	0.007
	Dumontiaceae								
		Constantinea sitchensis	94	10/vi	Several	(a); between tides	0.500	0.000096	0.019

These samples were all preserved in absolute alcohol, except Nos. 71 to 73, which were preserved in 10 per cent formal.

The sample of diatoms contained 80–85 per cent of *Melosira* and not more than 1 per cent of non-diatomaceous material. The material was obtained by filtering a large amount of surface water from Departure Bay. The absence of iodine is unexpected, and somewhat striking. Should further analyses of diatom material yield similar results it will follow that diatoms play no part in the cycle of iodine in marine life.

The conclusion that all Green, Brown, and Red Algae contain amounts of iodine greater than 0.001 per cent is confirmed by the new data. No Green Algae so far examined contain appreciably large amounts of iodine. This is not improbably connected with the fact that most of these species grow in the tidal zone.

Of the Brown Algae examined only the Laminariaceae contain marked amounts of iodine; none of the Red Algae recently examined contain appreciable amounts.

An attempt to discover variations in iodine content due to exposure was not successful. On August 20 numerous samples of a number of species of Algae were taken from a sloping rock on the north side of Jesse Island, an islet in Departure Bay. The specimens were growing under precisely similar conditions except as regards height above low water mark, and consequently degree of exposure to the atmosphere. In the following table the height above low water mark is given approximately by considering the rock surface divided by contour lines one foot apart vertically.

Species.	1st foot.	2nd foot.	3rd foot.	5th foot.	7th foot.	9th foot.	13th foot.	14th foot.
(Chlorophyceae)								
Monostroma fuscum	0.006(10)	0.002(11)	0.015(12)		0.029(13)			
Codium fragile					0.003(20)			
(Phaeophyceae)								
Desmarestia ligulata			0.004(23)					
Leathesia diformis	0.009(26)	0.011(27)						
Fucus furcatus			0.017(80)	0.015(81)	0.027(82)			0.011 (83)
Fucus evanescens				0.016(85)	0.019(86)	0.014 (87)	0.016 (88)	0.011 (89)
(Rhodophyceae)								
Chondrus crispus		0.009(90)						
Polysiphonia tubulata	0.007(93)							

The figures given are percentages of iodine, the numbers in brackets being the sample numbers of the specimens.

Figures for the same species show no regularity; the irregularity is greater than the error of analysis. The results illustrate chiefly the variation of the individual plant, if not of the individual cell. They are all low. All the high values of iodine content found are for plants growing below low tide mark.

More definite conclusions can be drawn from comparison of the same species growing under the same conditions, but collected at different times of the year. The following data are available from Parts I and II of this paper (figures for *Nereocystis* will be considered separately). The figures are percentages of iodine. The material was all gathered in Departure Bay.

Species.	August, 1913.	June, 1914.	August, 1914.
Laminaria bullata.....	0.060	0.270 (young plants) 0.175 (old plants)	
Laminaria saccharina.....	0.156 (small plants) 0.176 (medium sized plants)	0.206 (young plants) 0.078 (old plants)	
Fucus furcatus.....	0.015 (average)	0.042	0.017 (average)
Fucus evanescens.....	0.016 (average)	0.028	0.015 (average)

These figures show the effect of age (*Laminaria bullata* and *saccharina*) and a distinct effect of period of year, even in plants which are not annuals. The results agree with Scurti's data for *Sargassum* and *Cystoseira* (compare Part I, p. 353), as do those for *Nereocystis*. Before considering the latter in detail some determinations of the water content of the Laminariaceae will be given. At the beginning of the experiments an accurate balance was not available, so that some of the figures are less accurate. The samples were allowed to drain for about an hour before being weighed. The somewhat sticky surface of most of the Laminariaceae prevents the adherence of much water, so that error from this source is very slight. The material was all heated at 100°C. to constant weight.

Complete parts of the plant were taken in preparing Nos. 35 to 50, 52 to 54, 57, 58, of *Nereocystis*. The other analyses of *Nereocystis* are of carefully prepared samples. Ash determinations of *Nereocystis* were made in the case of a single plant. They

Species.	Sample No.	Weight fresh.	Weight dry.	Water.
		gm.	*gm.*	*per cent*
Laminaria bullata				
(young plants)	30	3.525	0.474	86.55
(old plants)	31	7.368	1.208	83.60
Laminaria saccharina				
(young plants)	32	3.350	0.419	87.49
(old plants)	33	7.263	1.732	76.15
Costaria turneri	34	7.613	0.933	87.75
Nereocystis lütkeana				
I frond	35	4.14	0.28	93.2
I float	36	0.80	0.11	86.3
I stipe	37	0.69	0.07	90.
II frond	38	4.95	0.32	93.6
II float	39	1.35	0.08	94.
II stipe	40	0.73	0.07	90.
III frond	41	12.20	0.82	93.3
III float	42	3.09	0.17	94.5
III stipe	43	1.61	0.16	90.
IV frond	45	23.7	1.74	92.7
IV float	46	2.50	0.14	94.4
IV stipe	47	1.65	0.14	91.5
V frond	48	29.4	2.58	91.2
V float	49	8.15	0.48	94.1
V stipe	50	2.90	0.40	86.2
VI frond	52	1.25	0.08	94.
VI float	53	0.65	0.04	94.
VI stipe	54	0.60	0.06	90.
VII frond	56	46.1	3.43	92.5
VII float	57	16.2	1.06	93.5
VII stipe	58	13.5	1.65	87.8
X frond	65	7.196	0.581	91.93
X float	66	7.355	0.447	93.92
X stipe	67	6.536	0.826	87.36
X holdfast	68	4.894	0.627	87.19
XIII frond	August, '13	5.27	0.568	89.2
XIII float	"	6.47	0.365	94.4
XIII stipe	"	6.90	0.541	92.2
XIII holdfast	"	3.38	0.258	92.4
Fucus furcatus	79	8.118	2.540	68.71
Fucus evanescens	84	8.070	1.628	79.83

are only approximate, since some inorganic salt was vaporized before the carbon was completely ignited.

No. 60 contained 44.5 per cent ash, No. 61 contained 49.9 per cent, and No. 62 contained 29.1 per cent.

The data for *Nereocystis* are shown in the following table. The length of the plant gives an idea of the degree of development. Most of the specimens were collected on the same day and at the same place.

No.	Where obtained.	Date.	Total length.	Iodine.			Water.			Ash.		
				Frond.	Float.	Stipe.	Frond.	Float.	Stipe.	Frond.	Float.	Stipe.
				per cent			*per cent*			*per cent*		
VI	(a)	26/v	8 in.				94	94	90			
I	"	"	1.3 ft.	0.272	0.20	0.305	93.2	86.3	90			
II	"	"	1.5 "	0.249	0.19	0.263	93.6	94	90			
III	"	"	2.0 "	0.257	0.086	0.305	93.3	94.5	90			
IV	"	"	2.1 "	0.274	0.111	0.210	92.7	94.4	91.5			
V	"	"	3.0 "	0.174	0.145	0.269	91.2	94.1	86.2			
VII	"	"	6.0 "	0.216	0.262	0.275	92.5	93.5	87.8			
VIII	"	27/v	11.0 "	0.288	0.196	0.251				44.5	49.9	29.1
IX	"	"	12.0 "	0 113	0.058							
X	"	10/vi	Full grown	0.250	0.130	0.133	91.9	93.9	87.4			
(1913)	"	August	Small size	0.184	0.120	0.147						
"	"	"	Full grown	0.171	0.090	0.161						
"	"	"	"				89.2	94.4	92.2			
"	(d)	"	Small size	0.064	0.217	0.085						
"	"	"	Full grown	0.130	0.108	0.046						
"	(f)	"	Small size	0.160	0.011							
XI	(j)	8/vii	Full grown	0.098	0.194							
XII	(o)	25/vii	"	0.079	0.167	0.065						

Careful examination of these figures shows that the percentage of iodine is almost invariably less, and the percentage of water greater in the float than in either the fronds or stipe. The ash determinations show a similar difference. The iodine content appears to diminish with growth, the highest values for frond and stipe

being obtained for the smallest plants. The water content of frond and stipe shows diminution with age (especially the latter), while that of the float is very constant. There is therefore an evident and marked difference between the composition of the float and that of the stipe.

From the fact that young plants of *Nereocystis* usually contain more iodine than full grown ones it follows that plants obtained during spring, when the majority are not full grown, will give a greater average yield of iodine than plants obtained later in the year. Comparison of the figures given for full grown plants with those quoted from other observers (Part I, p. 350) for plants from other localities does not reveal any differences more marked than those in the table itself, and does not give any definite evidence that latitude is a factor in iodine content of *Nereocystis* (compare Part I, p. 354).

The difference of iodine content in the same species growing under the same conditions, illustrated throughout the above tables, suggests that the individual plant cell has a particular action in retaining iodine within certain limits determined by the species.

Iodine Content of Animals.

Protozoa.

On the evening of May 13 the surface water of the bay at the Station was colored distinctly red by protozoa consisting almost entirely (98 per cent) of *Prorocentrum*. Five liters of water gave a small amount of residue consisting entirely of ciliate protozoa. 0.0809 gram of this material was analyzed, and was found to contain an unmeasurable trace of iodine.

Metazoa.

Phylum *Porifera*.—A specimen of (*Monaxonida*) *Esperiopsis quatsinoensis* (Lambe) was obtained on False Narrows reef at very low tide. 0.500 gram contained 0.000137 gram of iodine, equal to 0.027 per cent (dried material).

Phylum *Coelenterata*.—Through the kindness of Dr. A. Willey I obtained a specimen of a Verticillate fan coral, dredged in

Alaskan waters, and stated by Professor Hickson to be a Prinicoid coral, probably belonging to the genus *Caligorgia*. The coral was air-dried; three samples were examined, one of the whole coral, a second of the horny skeletal substance, and a third of the calcareous nodules surrounding the skeleton.

Material.	Sample No.	Amount taken.	Iodine found.	Iodine.
		gm.	*gm.*	*per cent*
Whole coral	97	0.500	0.000287	0.057
Skeleton	98	0.500	0.000493	0.099
Calcareous nodules	99	0.500	0.000009	0.002

Phylum *Vermes*, sub-phylum *Annulata*, class *Chaetopoda*, order *Polychaeta*.—Before dealing with the analyses, it is perhaps desirable to give some account of the nature of the worm-tubes.

The *Diopatra* worm-tubes consist of an upper part, 4 to 6 inches in length, covered with shells and small Algae, and a lower part, up to 18 inches in length, of parchment-like consistency, consisting of concentric layers, the inner being translucent and usually perfect, the outer more or less damaged. The lower tube is secreted by the glands of Tori, the leathery upper tube in part is a lip secretion. The tubes taken for examination were separated from adhering material (shells, Algae) and sand as far as possible, resolved into layers, and air-dried.

Only one specimen of *Onuphis* was obtained. The tube consisted of an inner hyaline layer, surrounded by an outer rigid cylinder, made up of very small pieces of rock cemented together.

The *Nerine* tubes consisted of a very thin collapsible membrane surrounded by a much thicker layer of sand, so that the whole was rigid. As much of the sand was removed as possible, by crumbling the tubes between the fingers, but a considerable proportion remained.

The *Chaetopterus* tubes had a similar structure to those of *Diopatra* but were thickly encrusted with sand. They were separated into layers, air-dried, and as much of the sand was removed as possible before bottling for transit.

The *Sabellaria* tubes consisted of mud, cemented together. The *Pallasia* tubes were similar, but included small stones.

Sub-order.	Family.	Species.	Where obtained.	Date.	Part examined.	Sample No.	Amount taken.	Iodine found.	Iodine.
							gm.	*gm.*	*per cent*
Rapacia									
	Eunicea								
		Diopatra (? californica)	(g); very low tide	12/v	Worm	100	0.500	0.000399	0.080
					Inner tube-layers	101	0.500	0.000612	0.122
					Intermediate tube-layers	102	0.500	0.000630	0.126
							0.500	0.000655	0.131
								(Mean	0.128)
					Outer (leathery) tube-layers	103	0.500	0.000203	0.041
			(m); dredged	26/vi	Worm	104	0.100	0.000148	0.148
					Inner tube-layers	105	0.121	0.000058	0.048
					Outer tube-layers	106	0.500	0.000416	0.083
		Onuphis (? Sp.)	(m); dredged	26/vi	Tube	107	0.391	(Doubtful trace)	
Limivora									
	Spiodea								
		Nerine vulgaris	(h); very low tide	7/vi	Worm	108	0.105	0.000100	0.095
					Tube	109	0.393	0.000087	0

	Chaetopterida	Chaetopterus (? Sp.)	(d); low tide	29/v	Worm	110	0.501	0.000062	0.012
					Inner tube-layers	111	0.500	0.002250	0.450
					Intermediate tube-layers	112	0.500	0.001667	0.333
					Outer tube-layers	113	0.500	0.001061	0.212
					Tube-ends	114	0.500	0.000478	0.096
		Phyllochaetopterus (? Sp.)	(i); dredged	20/vi	Tube	116	0.501	0.000256	0.051
		Spiochaetopterus (? Sp.)	(b); low tide	5/vii	Tube	117	0.373	0.000072	0.019
	Hermellacea								
		Pallasia saxicava	(i); dredged	19/vi	Tube	118	0.446	0.000038	0.008
		Pallasia saxicava and Sabellaria cementarium (mixed)	(i); dredged	19/vi	Tube	119	0.500	0.000093	0.019
	Amphictenia								
		Pectinaria (? Sp.)	(l); dredged	16/vii	Tube	120	0.340	0.000038	0.011
	Terebellacea								
		Amphitrite (? Sp.)	(g); very low tide	12/v	Worm	121	0.500	0.000056	0.011
					Tube	122	0.500	0.000196	0.039
		Thelepus (? Sp.)	(m); dredged	26/vi	Tube	123	0.0315	0.000090	0.28

Sub-order.	Family.	Species.	Where obtained.	Date.	Part examined.	Sample No.	Amount taken.	Iodine found.	Iodine
							gm	*gm*	*per cent*
Limivora									
	Ampharetea								
		Amphicteis (comes, M.S.)	(m); dredged	29/vi	Tube	124	0.311	0.000077	0.025
		Sabellides anops	(m); dredged	29/vi	Tube	125	0.501	0.000142	0.028
	Serpulacea								
		Sabella columbiana	(e); very low tide	8/vi	Worm	126	0.500	0.000159	0.032
							0.500	0.000139	0.028
					Inner tube-layers	127	0.500	0.003082	0.616
					Intermediate tube-layers	128	0.500	0.003028	0.606
					Outer tube-layers	129	0.500	0.002029	0.406
					Freshly secreted tube	130	0.111	0.000080	0.072
					Freshly secreted tube	131	0.123	None	0.00
			(a); between tides	11/vi	Worm	132	0.500	0.000299	0.060
					Inner tube-layers	133	0.500	0.002389	0.478
							0.500	0.002329	0.466
								(Mean	0.472)

					Outer tube-layers	134	0.500	0.002954	0.591
							0.500	0.002920	0.584
								(Mean	0.587)
			(n); low tide	26/viii	Worm	135	0.500	0.000211	0.042
					Tube	136	0.500	0.002363	0.473
			(a); between tides	2/ix	Worm	137	0.500	0.000284	0.057
					Tube	138	0.500	0.002859	0.572
		Bispira polymorpha	(g); very low tide	15/v	Tube	139	0.500	0.002977	0.595
			(a); between tides	15/vi	Worm	140	0.500	0.000267	0.053
					Inner tube-layers	141	0.500	0.002459	0.492
					Outer tube-layers	142	0.500	0.003705	0.741
			(a); between tides	2/ix	Worm (X)	143	0.500	0.000128	0.026
					Worm (Y)	144	0.300	0.000141	0.047
					Tube (X)	145	0.500	0.003281	0.656
					Tube (Y)	146	0.500	0.003511	0.702
							0.500	0.003468	0.694
								(Mean	0.698)
		Serpula columbiana	(a); between tides	8/v	Worm	147	0.500	0.000186	0.037
					Tube	148	0.500	0.000056	0.011
							0.500	0.000064	0.013
								(Mean	0.012)

Sub-order.	Family.	Species.	Where obtained.	Date.	Part examined.	Sample No.	Amount taken.	Iodine found.	Iodine.
							gm.	*gm.*	*per cent*
(?)	Phoronida								
		Phoronis vancouverensis	(a); between tides	27/v	Worm	149	0.500	0.000027	0.005
					Tube	150	0.301	0.000212	0.070
		Phoronopsis harmeri	(d); low tide	29/v	Worm	152	0.500	0.000018	0.004
					Inner tube-layers	153	0.136	0.000037	0.027
					Outer tube-layers	154	0.500	0.000048	0.010
							0.500	0.000045	0.009
								(Mean	0.009)

The *Pectinaria* tubes were thin, and of papier maché appearance. The *Amphitrite*, *Amphicteis*, and *Sabellides* tubes all consisted of mud, cemented together by a secretion.

The *Sabella* and *Bispira* tubes were tough, and horny in appearance, and consisted of numerous concentric layers of translucent material. They were resolved into several layers, and air-dried. They contained only very small amounts of inorganic material (some occasional patches which appeared to consist of calcium carbonate). The *Serpula* tubes consisted chiefly of calcium carbonate.

The *Phoronis* tubes were of thin hyaline material; those of *Phoronopsis* were thickly encrusted with sand, which was removed as far as possible after they had been air-dried.

Of the material given in the table, samples Nos. 100, 104, 108, 109, 110, 118, 119, 120, 121, 122, 126, 130, 131, 132, 135, 137, 140, 143, 144, 147, 149, 150, 152, 153, 154, were preserved in absolute alcohol; the remainder were air-dried. In each case several specimens were taken except for Nos. 104 (2), 107 (1), 126 (1), 132 (1), 133 (2), 134 (2), 137 (3), 139 (2), 140 (2).

All the worm-tissue examined, both above and in Part I of this paper, contains appreciable quantities of iodine, the limits observed being 0.004 and 0.148 and the average figure about 0.04 or 0.05 per cent. All the worm-tubes contain iodine, the observed limits being 0.009 and 0.741 per cent. The following table affords evidence that the iodine is in organic combination in the tubes, and that if a correction for inorganic material be applied the limits are much closer. The ash present in a number of samples was determined by ignition until constant weight was attained.

Qualitative examination of the ash showed that of the worm tissue, No. 100 contained practically no sand, and was almost completely soluble in hot dilute hydrochloric acid; No. 110 contained a little sand (in agreement with the higher percentage); No. 144 contained no sand, but some calcium; and No. 152 contained no sand, but was almost completely insoluble in hydrochloric acid (a black residue). The greater part of all the ash from the tube material consisted of sand grains, except that of Nos. 141, 142, and 146 in which very few sand grains were present, and No. 116 which contained a small amount only. This is in agreement with the observations on the nature of the tube material recorded above.

Species.	Part examined.	Sample No.	Amount taken.	Weight after ignition.	Ash.	Iodine in original material.	Iodine corrected for ash.
			gm.	*gm.*	*per cent*	*per cent*	*per cen*
Diopatra californica	Worm	100	0.500	0.042	8.4	0.080	0.087
Chaetopterus (? Sp.)	"	110	0.500	0.095	19.0	0.012	0 015
Bispira polymorphis	"	144	0.491	0.036	7.3	0.047	0 051
Phoronopsis harmeri	"	152	0.500	0.038	7.6	0.004	0.004
Diopatra californica	Inner tube-layers	101	0.500	0.180	36.0	0.122	0.191
	Intermediate tube-layers	102	0.500	0.221	44.2	0.128	0.229
	Outer tube-layers	103	0.500	0.393	78.6	0.041	0.192
Chaetopterus (? Sp.)	Inner tube-layers	111	0.500	0.201	40.2	0.450	0.754
	Intermediate tube-layers	112	0.500	0.285	57.0	0.333	0.774
	Outer tube-layers	113	0.500	0.362	72.4	0.212	0.768
	Tube-ends	114	0.500	0.438	87.6	0.096	0.774
Phyllochaetopterus (? Sp.)	Tube	116	0.046	0.024	52.	0.051	0.106
Bispira polymorphis	Inner tube-layers	141	0.117	0.020	17.1	0.492	0.593
	Outer tube-layers	142	0.442	0.074	16.8	0 741	0.891
	Tube	146	0.500	0.080	16.0	0.698	0.831
Phoronopsis harmeri	Tube	154	0.500	0.480	96.0	0.009	0.220

The data for *Diopatra* and *Chaetopterus* tube material sho that iodine content is strictly proportional to organic materi in the different layers of these tubes, and lend therefore stron evidence to the hypothesis that the iodine is in organic combin tion. The data suggest, since in most cases where small amoun of iodine were found for worm-tubes there was a considerab amount of inorganic material present, that the limits of iodin percentage, after correction for inorganic material, are probabl all within 0.1 and 1.0 per cent.

Since these tubes are secretions, and since the worms themselv contain iodine in comparable amounts, it would appear to follo naturally that the iodine compound present in the tube is secrete by the worm, although the hypothesis might be put forward th it was produced by some action of the iodine in sea water on t tube after secretion. The differences observed in the inner an outer layers of *Bispira* tubes (Nos. 133 and 134, and 141 and 14 although sea water has equal access within and without t tubes, seem to emphasize the action of the worm itself in pr ducing the iodine compound. Similar evidence is provided b the analyses of Nos. 130 and 131. No. 130 was from fresh tu

material secreted by two *Sabella* worms removed from their tubes and placed in sea water in the laboratory. The time of secretion was twelve hours. It will be observed that the percentage of iodine is much smaller than in the original tubes (0.6), while the further twenty-four hours' secretion (No. 131) contained no trace of iodine, proving that the iodine in the first secretion must itself have been secreted.

The good agreement in the corrected figures for No. 101 (pure secretion of glands of Tori in *Diopatra*) and 103 (mixed secretions of glands of Tori and lip) suggests either that the lip secretion is relatively small in amount, or that the two secretions contain similar quantities of iodine.

The *Bispira* worms showed a considerable degree of variation in the color of the tentacles. Those marked X were lake in color; those marked Y were lighter, varying from pale pink to salmon color. There is some indication of a corresponding difference in iodine content.

The figures, taken as a whole, do not show any definite alteration of iodine content at different periods of the year (this would scarcely be expected for the tube material). In Part I it was shown that *Serpula* tube material, from which the calcium carbonate had been removed, contained iodine of the order of 0.7 per cent, and it therefore appears that the closely related species of the *Serpulacea* contain very similar quantities of iodine (both worms and tubes).

Phylum *Mollusca*.—The presence of iodine in appreciable quantity in the dermis of the "foot" of *Schizothoerus (Treseus) nuttalli* has been confirmed. Some other secreted material has been examined in other species.

The dermis of the foot of the horse-clam is stated to be a secretion of the sub-dermis. The new figures confirm the result in Part I (p. 362) that the dermis contains marked quantities of iodine, showing a considerable concentration during the secretion. The foot of the clam shows no marked division between dermis and sub-dermis; no separation could be effected and the figure obtained resembles that for the sub-dermis of the horse-clam. Considerable individual variations are shown for the horse-clam (the figures in each case are for one or two specimens only)

Species.	Where obtained.	Date.	Part examined.	Sample No.	Amount taken.	Iodine found.	Iodine.
					gm.	*gm.*	*per cent*
Cardium corbis (cockle)	(e); low tide	8/vi	Dermis of foot	155	0.105	0.000248	0.236
Saxidomus gigantica (clam)	(e); low tide	8/vi	Dermis of foot	156	0.500	0.000045	0.009
Schizothoerus nuttalli (horse-clam)	(d); low tide	30/v	Dermis of foot	158	0.500	0.000462	0.092
			Sub-dermis	157	0.300	0.000038	0.013
	(e); low tide	8/vi	Dermis	160	0.500	0.000795	0.159
			Sub-dermis	159	0.500	(Doubtful trace)	
	(h); low tide	9/vi	Dermis	162	0.500	0.000514	0.103
			Sub-dermis	161	0.500	0.000016	0.003
Mytilus edulis (mussel)	(a); between tides	25/vi	Byssus	163	0.500	0.000222	0.044
					0.500	0.000199	0.040
						(Mean 0.042)	
Polynices lewisii (whelk)	(d); low tide	28/v	Opercula	240	0.500	0.000146	0.029
					0.500	0.000160	0.032
						(Mean 0.030)	
		viii/13	Egg-case	243	0.500	0.000051	0.010

and none of the figures are as high as that previously recorded. The byssus of *Mytilus* is an adhesive secretion, the opercula of *Polynices* a protective secretion. The egg-case of *Polynices* consists of sand grains cemented together by a secretion.

Nos. 240 and 243 were air-dried; the other material was preserved in alcohol.

Phylum *Chordata*, sub-phylum *Tunicata*.—A large number of tunicates have been examined. The classification followed is that of Huntsman.[12]

These results (pages 28-31) show that iodine is an invariable constituent in the test of the ascidian, the amount varying markedly in different species; the limits observed are from a trace to 0.3 per cent. The figures show a definite differentiation between species but not between families. The figures for different individuals of the same species, living under precisely the same conditions (*Pyura haustor*) show a considerable variation (0.09

[12] Huntsman, *Contributions to Canadian Biol.*, 1912, 103.

to 0.3 per cent), and this is not due to variation in inorganic constituents, since the ash was determined in the samples giving these extreme values and is not very different in the two cases:

No. 202 contained 42.3 per cent ash, giving a corrected iodine value 0.516 per cent.

No. 204 contained 51.5 per cent ash, giving a corrected iodine value 0.193 per cent.

The data presented are insufficient to show any definite variation in the content of the element in different specimens of the same species from different localities, especially in view of the variation shown in specimens from the same locality. The highest values are found for animals living just below the tidal zone (*Pyura*) and not for dredged material.

The inner test, attached throughout to the test, and sometimes not separable, consists apparently chiefly of connective tissue (mesoderm). The amount of iodine present in the inner test is usually not detectable. It is found present for *Pyura;* in this species the content of the outer test is large. The same kind of relation appears to hold for the animal removed from the test. The *Pyura* material contains a definite but very small amount; the result for *Ascidiopsis paratropa* is almost certainly an error, and that for *Tethyum igaboja* is doubtful, but the material was insufficient for duplicate analyses. The results for other species were negative.

Considerable evidence has been put forward that the endostyle is closely related to the thyroid. The endostyles examined were dissected from surrounding tissue as carefully as possible, but the contamination with non-endostyle tissue was undoubtedly large. The results obtained suggest, however, that the endostyle does not perform a function similar to thyroid as regards iodine.

The whole of the ascidian material was preserved in absolute alcohol.

Family.	Species.	Where obtained.	Date.	No. of specimens.	Part examined.	Sample No.	Amount taken.	Iodine found.	Iodine.
							gm.	*gm.*	*per cent*
Phalusiidae									
	Ascidiopsis (? columbiana)	(a); dredged	29/vi	6	Test	164	0.395	0.000053	0.013
	Ascidiopsis paratropa	(a); "	"	1	Test	167	0.203	0.000011	0.005
		(i); "	20/vi	2	Test	168	0.260	0.000025	0.010
		(c); "	3/vii	2	Test	169	0.250	0.000012	0.005
		(a); "	29/vi		Animal minus test and endostyle	170	0.1794	0.000248	0.138
				Several	Endostyle	171	0.0088	None	0.0
				"	Mantle minus endostyle	172	0.0284	None	0.00
	Phallusia ceratodes	(a); dredged	29/vi	7	Test	174	0.250	0.000319	0.128
							0.346	0.000398	0.115
								(Mean	0.120)
				7	Animal	173	0.311	None	0.000

Chelyosomatidae									
	Corella rugosa	(a); low tide	14/v	11	Test	175	0.153	0.000026	0.017
		(i); dredged	19/vi	8	Test	178	0.092	None	0.00
		Mixed sample, (e); low tide, 8/vi, and (m); dredged, 28/vi			Test	180	0.329	Trace	Present.
					Animal	179	0.342	None	0.000
	Chelyosoma productum	(e); low tide	8/vi	5	Test	182	0.113	0.000075	0.066
				5	Animal	181	0.141	None	0.00
		(m); dredged	28/vi	9	Test	184	0.405	0.000742	0.184
				9	Animal	183	0.500	Doubtful trace.	
Caesiridae									
	Caesira apoploa	(i); dredged	19/vi	2	Test	185	0.0064	None	0.0
Styelidae									
	Styella gibbsii	(m); dredged	26/vi	12	Test	188	0.500	0.000585	0.117
				12	Inner test	189	0.0400	None	0.00
				12	Animal	187	0.380	None	0.000
	Goniocarpa coccodes	(a); dredged	29/vi	4	Test	190	0.0449	Trace	Present.
	Cnemidocarpa joannae	(a); low tide	14/v	13	Test	190A	0.250	0.000264	0.106
				13	Inner test	191	0.084	None	0.00
		(e); low tide	8/vi	7	Test	193	0.302	0.000120	0.040
				7	Inner test	194	0.029	None	0.00
				7	Animal	192	0.500	None	0.000
Tethyidae									
	Boltenia arctica	Mixed sample; dredged		4	Test	195	0.0520	0.000158	0.304

Family.	Species.	Where obtained.	Date.	No. of specimens.	Part examined.	Sample No.	Amount taken.	Iodine found.	Iodine.
							gm.	*gm.*	*per cent*
Tethyidae									
	Boltenia villosa	(a); low tide		5	Test	196	0.243	0.000203	0.083
		(e); low tide		1	Test	199	0.250	0.000530	0.212
		Mixed sample		6	Inner test	200	0.0410	None	0.00
		(e); low tide		1	Animal	198	0.108	None	0.00
	Pyura haustor	(g); very low tide	14/v	1	Test	201	0.500	0.001079	0.216
				1	Test	202	0.500	0.001512	0.302
							0.500	0.001476	0.295
								(Mean	0.298)
				1	Test	203	0.500	0.001010	0.202
				1	Test	204	0.500	0.000495	0.099
							0.500	0.000451	0.090
								(Mean	0.094)
				1	Test	205	0.500	0.001234	0.247
				60	Inner test	213	0.501	0.000054	0.011
				5	Animal	207	0.500	0.000021	0.004
							0.353	0.000015	0.004
								(Mean	0.004)
				8	Mantle and endo-style	208	0.500	None	0.000

				17	Part of mantle and branchial sac surrounding endostyle	209	0.379	None	0.000
				17	Endostyle	210	0.091	None	0.00
		(h); very low tide	6/vi	2	Test	216	0.500	0.000134	0.027
	Tethyum aurantium	(m); dredged	28/vi	1	Test	218	0.250	0.000052	0.021
				1	Animal	217	0.362	0.000010	0.003
	Tethyum igaboja	(c); dredged	3/vii	1	Test	220	0.260	0.000439	0.169
				1	Animal	219	0.145	0.000051	0.035
		(k); dredged	9/vii	1	Test	221	0.081	0.000103	0.127

Some rough determinations of the water percentage were and may be conveniently given here.

Species.	Part examined.	Sample No.	Weight fresh.	Weight dry.	
			gm.	*gm.*	
Corella rugosa.....	Test	175	4.3	0.16	
Cnemidocarpa joannae.....	Test	190A	6.8	1.00	
	Inner test	191	1.3	0.09	
Boltenia villosa.....	Test	196	2.4	0.25	
Pyura haustor.....	Test	201	7.0	2.06	
	Test	202	10.0	1.84	
	Test	203	7.5	1.29	
	Test	204	8.3	1.73	
	Test	205	7.0	1.63	
	Inner test	206	2.0	0.21	
	Animal	207	80.	2.30	

The samples Nos. 178 to 180 may have contained so *inflata* mixed with the *C. rugosa*.

Phylum *Chordata*, sub-phylum *Vertebrata*.—In the ser analyses of the tissues of the dog-fish *Squalus sucklii* publis Part I of this paper (p. 373) the egg-case was not inc Some egg-cases of the skate and rat-fish were obtained by ing last summer, and the analyses are appended (the m was air-dried, and then dried at 100° C.):

Species.	Material.	Amount taken.	Iodine found.	Io
		gm.	*gm.*	pe
Skate (Sp. ?).....	Egg-case	0.500	0.001090	0
		0.500	0.001166	0
				(Mea
Hydrolagus collieii (rat-fish).....	Egg-case	0.500	0.000146	0

These results were unexpected and suggest further exa tion of such material.

Thyroid Gland.—The fish thyroids in the following tabl obtained by local fishermen in Departure Bay and the vi

except Sample No. 228, obtained off Rose Spit, north of the Queen Charlotte Islands. This sample was preserved in formal, the others were preserved in absolute alcohol. The crow thyroids were from birds shot near the Station; the other bird material was shot at points inside the Strait of Georgia. The cougar thyroid was from a young adult.

The weights of animals, where expressed in brackets, were calculated from those of a number of individuals selected at random. In other cases all the animals used were weighed.

The varying relation existing between amount of thyroid tissue and body weight in the different families of the Vertebrata, pointed out in Part I (p. 368) is supported by the constant figures for *Squalus*, the definitely higher figure for the holocephaloid fish, and the still higher figures for birds. It does not seem accidental that the figure for Sample No. 231 which is much higher than that for the other rat-fish samples, corresponds to a higher water content and a low (though not the lowest) iodine percentage, suggesting that this sample contained some goitrous material. Such goitrous material has recently been observed in *Squalus*.[13]

The mean average iodine content for the thyroids of male *Squalus* from the Departure Bay district is 0.208 per cent, that for females, 0.166. This agrees with the higher results for males previously found (Part I, p. 364) and somewhat emphasizes the difference between *Squalus* and mammals, for which Fenger has shown that usually the female thyroid contains the higher percentage.[14] The mean found for all the analyses of *Squalus* obtained in May (0.185) is not far removed from that for *Squalus* obtained in August (0.200, Part I, p. 364). The figure for *Squalus* from the open ocean (0.165 per cent) is probably at least 10 per cent too small, since a special test to show the effect of evaporation with formal, carried out with Merck's "thyroidin" gave a figure of 0.343 for material containing 0.388 per cent iodine, showing a loss of 11.6 per cent. It would appear therefore that the iodine content of the thyroid of *Squalus* is remarkably constant (when large numbers of fish are taken) both at different seasons

[13] Cameron, A. T., and Vincent, S., *Jour. Med. Research*, 1915, xxxii, 251.

[14] Fenger, *Jour. Biol. Chem.*, 1911–12, xi, 489; 1913, xiv, 397.

Species.	Sex.	Date.	No. of animals.	Weight of animals.	Sample No.	Weight of fresh thyroid.	Weight of dry thyroid.	Water.	Dry thyroid tissue per kg. animal.	Amount taken for analysis.	Iodine found.	Iodine.
				kg.		*gm.*	*gm.*	*per cent*	*mg.*	*gm.*	*gm.*	*per cent*
Pisces												
Elasmobranchii												
Squalus sucklii	m	11/v	14	32.2	222	1.67	0.245	85.3	7	0.245	0.000462	0.189
(dog-fish)	f	"	3	11.4	223	0.47	0.071	84.9	6	0.0711	0.000168	0.236
	m	19/v	61	(140)	224	5.19	0.836	83.9	6	0.200	0.000432	0.216
	f	"	34	(177)	225	6.30	1.030	83.7	6	0.200	0.000372	0.186
										0.200	0.000370	0.185
											(Mean	0.185)
	m	20/v	4	8.6	226	0.24	0.045	81.3	5	0.0442	0.000072	0.16
	f	"	12	53.5	227	0.37	0.390	83.5	7	0.332	0.000345	0.104
									(Mean 6)		(*Mean of*	
											above	*0.185*)
		29/vii	6	12	228		0.092		7.5	0.0925	0.000153	0.165
Raja rhina	m	23/v	1	2.7	229		0.013		5	0.0100	0.000031	0.3
Holocephali												
Hydrolagus collieii		11/v	6	4.7	230	0.27	0.048	82	10	0.0450	0.000336	0.747
(rat-fish)		19/v	4	3.2	231	0.71	0.067	91	21	0.067	0.000391	0.584
	m	20/v	17	7.7	232	0.21	0.051	76	7	0.0500	0.000310	0.620
	f	"	10	8.5	233	0.55	0.105	80	12	0.1050	0.000553	0.527
	f	23/v	7	7.4	234	0.51	0.099	80	13	0.0989	0.000689	0.697
									(Mean 12)		(Mean	0.623)

Aves										
Corvus corvinus (crow)	June	9	2.9	235	0.079	27	0.0782	0.000587	0.751
Oedemia perspicillata (surf-scoter)	August	2	2.0	236	0.071	35	0.0687	0.000783	1.14
Uria arra (pigeon guillemot)	"	2	1.0	237	0.021	21	0.0157	0.000062	0.39
(?) Marble murrelet	"	7	2.5	238	0.044	17	0.0395	0.000124	0.31
Mammalia									
Felis concolor (cougar)	June	1	.	239			0.134	0.000521	0.389

of the year and from different localities. In order to explain the marked difference in the figures for *Squalus* and *Scyllium* (compare Part I, p. 369), it is necessary to assume a constant difference of diet for the two species or a specific variation in thyroid tissue as regards iodine. The former assumption appears more likely.

The high figures for *Hydrolagus* indicate a very different diet for this species. (The position of the thyroid in *Hydrolagus* is the same as in *Squalus;* its appearance is more like that of the skate than of the dog-fish thyroid.)

The only definite figure previously published for bird thyroids is that for pigeons (0.485, see Part I). The values for other species given above are all of the same order. The high value for the crow is undoubtedly due to the fact that the birds examined had been feeding largely on clams and other shell-fish. While the value for the surf-scoter is based on an analysis of a very small amount of material and requires confirmation, it is somewhat striking that the three highest values obtained for fish, birds, and mammals are all approximately equal, and equal to the highest value obtained by actually feeding iodide to sheep. This agreement seems more than accidental.

The revised figures for the maximum percentages of iodine so far found in different species are:

Fish thyroids (Scyllium) 1.16 per cent (Cameron); bird thyroids (Scoter) 1.14 per cent (Cameron); sheep thyroids 1.05 per cent (Hunter and Simpson); dog thyroids 0.692 per cent (Marine and Lenhart); human thyroids 0.588 per cent (Seidell); stag thyroids 0.54 per cent (Blum); pig thyroids 0.531 per cent (Seidell and Fenger); beef thyroids 0.477 per cent (Marine and Lenhart). (Compare Part I, p. 367.)

The maximum figure for iodine in thyroid tissue after feeding iodide is 1.15 per cent (Simpson and Hunter).[15]

Reference was made at the beginning of this paper to Seidell and Fenger's theory that the seasonal variation observed by them in the iodine content of mammalian thyroids is due to a temperature effect; this temperature effect they consider is produced by an increased metabolism at high temperatures using up the iodine compound poured out from the thyroid and depleting

[15] Simpson and Hunter, *Quart. Jour. Exper. Physiol.*, 1911, iv, 257.

stored iodine; cold weather results in a reversed effect. Since merous data prove definitely that very slight variations in the ount of iodine fed at once affect the amount in the thyroid nd, while all the differences observed in different species can satisfactorily attributed to difference in diet, until more direct dence is adduced, it seems more natural to attribute the sea- al variation which undoubtedly occurs in several mammals changes in the iodine content of their diet, and not to intro- e a new, and in any case a merely additional factor.

SUMMARY OF RESULTS.

From the fresh data presented in Parts I and II of this paper, l from previously published data by others summarized therein, following conclusions can be drawn with some certainty.

odine is an invariable constituent of all marine Algae. The its observed in reliable analyses are 0.001 and 0.7 per cent.

the Brown Algae only the Laminariaceae (and one or two aceae) contain amounts of iodine greater than 0.1 per cent.

the Red Algae only the Rhodymeniaceae and Delesseraceae tain similar amounts. None of the Green Algae contain appre- bly large amounts. Almost all the species for which appre- ble quantities of iodine have been reported grow below the al zone and are never exposed. Young plants contain more ine than full grown plants. The amount of iodine present an individual plant is, within certain limits for each species, variable quantity apparently determined by the plant cells emselves. Iodine varies in different parts of the individual ant. In *Nereocystis* the float usually contains less of the ele- nt than either the fronds or the stipe. No definite relation- ip exists between the percentage in fronds and stipe, and no finite statement can be made as to which contains the larger ount. At least one species of diatom does not appear to con- n the element in detectable quantity.

Land plants contain very much less iodine, although it is widely tributed in them. They exhibit the same variations, showing lifferentiation not only determined by the species, but appar- ly by the plant cell also.

All sea species of animals contain iodine. As advances in evo- ion are made there is more differentiation and probably less

total iodine in the whole organism. Much of the material which has been found to contain marked quantities of iodine is in the nature of an external secretion (a secretion on to the outer surface of the body).

All sponges, corals, annelid worms (worm and tube) and ascidians (test) contain iodine. The amount varies very much in different species. The limits observed are very similar.

Appreciable quantities of the element have been found in the dermis of the foot of the horse-clam and related species, in the opercula of the whelk, and in the byssus of the mussel.

The endostyle of ascidians, if it contains iodine, contains a much smaller amount than the thyroid of other vertebrates.

Of vertebrate tissue the thyroid alone is of importance in connection with the storage of iodine. The limits in the amounts found in thyroid (dry) tissue are 0.01 and 1.16 per cent. Other tissues in mammals contain less than 0.001 per cent. In fish the liver and kidney may contain slightly higher amounts than this. (Anomalous results have been found for the egg-cases of certain fishes; these require further examination.)

Variations in the iodine content of thyroid tissue can all be traced to differences in diet.

While Fenger has adduced definite evidence that in mammals thyroid tissue of females contains more iodine than that of males, this does not appear to be the case in the thyroids of elasmobranch fish.

It is hoped to examine the annelid worm-tube and ascidian test material further with the purpose of finding the form of combination of iodine in these tissues.

In conclusion I have again to acknowledge my great indebtedness to Dr. C. McLean Fraser, the Curator of the Biological Station at Departure Bay, B. C., for his kindness in assisting me in the collection and identification of the material dealt with in this paper, and to Professor A. Willey, F.R.S., who identified all the annelid worms and gave me much other assistance. I wish also to thank Professor Swale Vincent for his continued interest and encouragement in this work.

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The work forms part of researches carried out under the direction of the Ductless Glands Committee of the British Association.

THE INFLUENCE OF ELECTROLYTES UPON THE DIFFUSION OF POTASSIUM OUT OF THE CELL AND INTO THE CELL.

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I. INTRODUCTION.

We intend to show in this paper that potassium cannot diffuse out of the egg of *Fundulus* when the latter is put into distilled water or into a saccharose solution, while potassiun can diffuse out from the egg easily when the latter is put into a solution of an electrolyte; and that the influence of electrolytes upon this diffusion increases with the valency of the anion of the salt and follows possibly the Hofmeister series of anions. We will also show that when we keep eggs for some time in H_2O and put them afterwards into a KCl solution a barrier is created which retards the entrance of potassium into the egg. These experiments have a bearing not only upon the mechanism of a certain group of phenomena of antagonistic salt action but also upon the mechanism which determines the diffusion of cations through cell walls. Certain theoretical aspects of these experiments have already been discussed in a previous paper.[1]

Loeb and Wasteneys have shown in 1911[2] that the marine fish *Fundulus* is rapidly killed by a pure KCl solution in that concentration of this salt in which it is contained in sea water, namely 2.2 cc. M/2 KCl in 100 cc. H_2O; while the fish live indefinitely when NaCl is added in such a ratio that the solution contains 17 molecules or more of NaCl for each molecule of KCl. Na_2SO_4 was approximately twice as effective as NaCl but the quantitative determinations were restricted by the great toxicity of

[1] Loeb, J., *Proc. Nat. Acad. Sc.*, 1915, i, 473.

[2] Loeb, J., and Wasteneys, H., *Biochem. Ztschr.*, 1911, xxxi, 450; 1911, xxxii, 155.

Na_2SO_4. Experiments on the possible antagonistic action of other salts than NaCl or Na_2SO_4 could not well be made since most salts were too toxic for the fish. Loeb and Wasteneys explained this result on the assumption that the presence of NaCl or Na_2SO_4 in the solution prevented the KCl from diffusing into the fish. Assuming that the KCl, in order to diffuse into the fish (*i.e.*, into the blood and thus reaching the heart and the central nervous system), had to undergo a combination with a colloidal constituent of the skin of the fish, the Na ions of the NaCl or Na_2SO_4 by competing with the K for the colloidal anion would prevent the latter from combining with this constituent.

Our new experiments were made on the embryo of *Fundulus* instead of on the adult fish. The embryo is surrounded by the rather thick egg membrane inside of which it moves freely. Any salt, before it reaches the embryo, must therefore diffuse through the membrane. When we put an egg containing an embryo from four to fourteen days old into a pure KCl solution until the heart of the embryo stops beating we know that the KCl must have diffused through the egg membrane into the egg in such quantity as to reach the toxic limit for the heart of the embryo; and if such an egg is afterwards put into a solution free from KCl, where its heart recovers, we may conclude that enough KCl must have diffused from the egg into the outside solution, so as to bring the concentration of KCl inside the egg below the limit required to cause the standstill of the heart.

II. THE INFLUENCE OF THE ANION IN THE PREVENTION OF THE TOXIC ACTION OF KCl.

When we try to investigate the prevention of the toxic action of KCl on the adult fish by other electrolytes we are restricted by the fact that only a few salts are sufficiently harmless for the fish to be used for such a purpose. In the case of the embryo, which is separated from the outside solution by the egg membrane, we have a much greater freedom in the choice of our antagonistic salts.

In the experiments to be mentioned in this chapter we ascertained the influence of the concentration of salts and the sign and nature of the ions upon the rapidity with which a given con-

centration of KCl caused the hearts of the embryos to stop beating. As a rule, twenty embryos of the same age whose hearts were beating were put into each solution and the number of embryos whose hearts were still beating was ascertained at various intervals.

We found first that if we add to a given concentration of KCl another salt, *e.g.*, Na_2SO_4, in various concentrations, the poisonous action of KCl upon the heart will be the more retarded the higher the concentration of Na_2SO_4. In one experiment of this kind 6.6 cc. M/2 KCl were contained in 50 cc. of the solution.

TABLE I.

After	Number of surviving hearts in 6.6 cc. M/2 KCl dissolved in 50 cc. of						
	H_2O	M/4	M/8	M/16	M/32	M/64	M/128 Na_2SO_4
hrs.							
1	14	20	20	20	15	10	11
4	5	20	17	11	11	8	9
9½	3	19	16	7	4	3	2
24	1	19	13	4	3	3	0
72	1	14	9	2	1	2	0

While after nine and a half hours practically all the hearts in M/4 Na_2SO_4 were still beating, in the solutions of Na_2SO_4 below M/32 and in distilled water only four or less of the original twenty were beating.

An experiment with Na acetate in different concentrations gave a similar result.

We next tried the effect of different sodium salts to find out whether the anion had any effect in this case. This was found to be true. 6.6 cc. M/2 KCl were dissolved in 50 cc. of the following solutions (Table II). Twenty embryos, five days old, were put into each of the solutions.

The experiment shows that the antagonistic effect of Na_2SO_4 and Na_2 tartrate is very powerful since after two days the hearts of almost all the embryos were still beating. Next in efficiency was the acetate which was found to be much more effective than NaCl. The antagonistic effect of a salt against KCl is therefore in this series a function of the anion and grows with the valency of the latter. The order of efficiency of the anions is:

SO_4, tartrate > acetate > Cl, Br, I.

This suggests to some extent the Hofmeister series of anic efficiency and this statement will find fuller corroboration in later chapter. We come, therefore, to the conclusion that KC causes the hearts of the embryo of *Fundulus* to stop beating mos rapidly when alone in solution, while sodium salts inhibit or re tard this effect, (a) the higher (within certain limits) their concer tration, and (b) according to the nature of the anion of the sa added, the order of increasing efficiency being

Cl, Br, I < acetate < SO_4, tartrate.

It is desirable to take for these experiments young embryos since in older embryos the time required for causing the stanc still of the heart by KCl is greater.

TABLE II.

After	Number of hearts beating in 6.6 cc. M/2 KCl in 50 cc. of								
	H_2O	M/4 sea water	M/4 NaCl	M/4 NaBr	M/4 NaI	M/4 Na acetate	M/4 NaCNS	M/4 Na_2SO_4	M/4 Na_2 tartrate
hrs.									
1	11	19	18	18	18	20	16	20	20
4½	2	12	14	11	16	15	3	19	19
22	1	11	11	8	3	13	1	18	16
48	1	7	9	4	1	14	0	18	16

These experiments confirm the older observations of Loeb an Wasteneys on the adult fish, but they add the fact of the rôle c the anion in this antagonism. This rôle could not be ascertaine on the adult fish since most of the salts, like tartrates an acetates, are too toxic for the adult fish to be used for antago nistic purposes.

We also confirmed the result found in the previous investiga tion,[3] that it was only possible to inhibit the poisonous action o KCl by other salts as long as the concentration of KCl did no exceed a certain limit.

[3] Loeb and Wasteneys, *Biochem. Ztschr.*, 1911, xxxi, 450.

III. THE IMPOSSIBILITY OF RECOVERY OF THE EMBRYO FROM KCl POISONING WITHOUT THE AID OF ELECTROLYTES.

Eggs with normal heart beat were put for several hours (usually three and a half) into a M/2 KCl solution to cause their hearts to stop beating.[4] They were then put into distilled water or different salt solutions to find out in which solution they recover most quickly. The most unexpected result obtained was that if the embryos had been sufficiently poisoned they would not recover at all or only in exceptional cases when put into distilled water or into a solution of saccharose, while they recovered rapidly when put into different salt solutions.

We will first discuss the fact that embryos whose hearts had stopped beating under the influence of a sufficient dose of KCl did not begin to beat when put into distilled water[5] or into a solution of saccharose, and that such eggs which had not recovered in distilled water after a number of days recovered in less than a day when put into a salt solution.

A number of eggs with embryos whose heart beat had developed were put into a M/2 KCl solution for three and a half hours. Sixty eggs whose hearts had stopped beating were distributed in two dishes, one containing sea water, the other distilled water. Table III gives the rate at which the eggs recovered.

TABLE III.

After	Number of hearts which began to beat in	
	Sea water	H_2O
hrs.		
1	0	0
2	2	0
3	7	0
4	11	0
5½	20	2
8½	27	2

[4] In a M/2 KCl solution the hearts stop beating in less than 3½ hours, but the results are clearer if the eggs contain an excess of KCl.

[5] The reader will remember that distilled water is harmless for these eggs and that the embryos of *Fundulus*, while marine organisms, will develop, hatch, and live in distilled water.

The next morning all thirty eggs had recovered in sea water and only three eggs in distilled water. The eggs which had not recovered in distilled water were then put into sea water, where they recovered at almost but not quite the same rate as those originally put into sea water (Table III), namely two after 2 hours, five after 4 hours, nine after 5½ hours, and twenty-two after 9 hours, had recovered and the next morning all had recovered, showing beating hearts.

A saccharose solution no more permits the recovery of the heart after KCl poisoning than does H_2O. Eggs that had been treated for three and a half hours with a M/2 KCl solution and whose hearts had stopped beating were put into H_2O, M/2, M/8, and M/32 saccharose, and into sea water, and the number of beating hearts was ascertained (Table IV). Twenty embryos were put into each solution.

TABLE IV.*

After	Number of hearts which began to beat after standstill in KCl, in				
	Sea water	H_2O	M/2 saccharose	M/8 saccharose	M/32 saccharose
hrs.					
1½	1	1	0	0	0
3	7	2	0	0	0
4½	13	2	0	0	0
7½	18	2	1	0	0
21½	20	2	2	0	0
47½	20	3	2	1	0
96	20	2	0	1	0

* Attention should be called to the fact that after 96 hours only two hearts were beating in H_2O, while three had been beating after 47½ hours. This observation is not uncommon in the recovery experiments and is due to the fact that a heart may beat for a little and then stop again without recovering permanently.

There was therefore in four days practically no recovery of the eggs which had been in H_2O or saccharose solutions, while the eggs put into sea water had almost all recovered in seven and a half hours.

The eggs that had not recovered in the H_2O or in the sugar solutions were not dead. In order to test this, those that had not recovered in H_2O and in M/32 saccharose in four days were put

into M/8 solutions of NaCl, Na_2SO_4, NaI, and NaCNS. In six hours all or the majority of the eggs recovered.

The question arose, how long can the eggs poisoned with KCl live in distilled water without recovering and without losing the power of recovering when put into a salt solution?

Twenty-one eggs were put for five and a half hours into a M/2 KCl solution and then transferred to H_2O, where they remained for seven days. During all this time only three hearts began to beat again. Nine of the remaining eighteen eggs were then put into M/8 Na_2SO_4; after eighteen hours the hearts were beating in every one of the nine eggs. The other nine eggs remained for five days longer in distilled water during which time none of them began to beat again. They were then put into normal sea water and in six hours the hearts were beating in six of these eggs, but the others did not recover even after a longer exposure. This experiment, therefore, shows that some of the eggs which had not recuperated from KCl poisoning in twelve days were still able to recover in a short time when put back into sea water. The circulation was not established in these extreme cases, which indicates that the long cessation of the heart beat is not entirely harmless if the temperature is high, as was the case in these experiments.

These and many similar experiments show that the recovery of the *Fundulus* egg poisoned with KCl is only possible in a salt solution and not in distilled water nor in a solution of saccharose (and probably other non-electrolytes).

Only when the KCl has acted but a short time can the hearts recover in H_2O or a saccharose solution.

IV. THE RELATIVE EFFICIENCY OF DIFFERENT SALTS FOR THE RECOVERY OF EGGS POISONED WITH KCl.

We have seen that eggs poisoned with KCl will recover quickly when put into a salt solution but will not recover in H_2O or a saccharose solution. When put into a salt solution eggs poisoned with KCl will recover the more quickly the higher (within certain limits) the concentration of the salt of the surrounding solution. One experiment each of recovery in Na_2SO_4 and NaCl of different concentrations may serve as examples. Both sets

of experiments were made simultaneously. A large number of eggs of the same age were put for three and a half hours into a M/2 KCl solution and those whose hearts had stopped beating were selected for the experiment. Twenty eggs were put into each of the following solutions of Na_2SO_4 and NaCl. As a control the recovery of such eggs in H_2O and in sea water was also noted (Tables V and VI).

TABLE V.

After	Number of hearts which began to beat in					
	M/4	M/8	M/16	M/32	M/64	M/128 Na_2SO_4
hrs.						
1	2	0	1	0	0	0
2	6	3	5	0	2	0
3	13	11	7	2	2	0
5	20	19	19	13	4	3
7½	20	20	20	18	11	8
19	20	20	20	20	19	17

TABLE VI.

After	Number of hearts which began to beat in						
	H_2O	Sea water	M/2	M/8	M/16	M/32	M/128 NaCl
hrs.							
1	0	1	1	0	0	0	0
2	0	3	4	3	0	0	0
3	0	6	10	4	1	0	0
5	0	10	20	17	7	2	0
7½	0	17	20	20	17	6	0
19	0	20	20	20	19	16	0

Several facts are worthy of notice. First, the tables show as stated that the hearts begin to recover the more quickly from the previous poisoning in KCl the more concentrated the solution. Second, a comparison of identical concentrations of NaCl and Na_2SO_4 shows that the eggs recover more quickly in Na_2SO_4 than in NaCl, and that the efficiency of Na_2SO_4 is *more* than twice as great as that of NaCl for equal concentrations, since a M/64 solution of Na_2SO_4 corresponds in efficiency to a M/16 solution of NaCl and a M/32 NaCl solution to a M/128 solution of Na_2SO_4.

The third fact worth noticing is that sea water is less efficient than NaCl of the same osmotic pressure. We were also surprised to find (in experiments which we shall not record here) that the addition of $CaCl_2$ to NaCl does not increase the inhibiting influence of the latter salt upon the diffusion of KCl into or from the egg.

We now wish to show that the effect of the salts upon the rate of recovery of the egg is influenced very strongly by the anion of the salt or salts added.

Eggs were put into a M/2 KCl solution for three hours, and those in which the hearts had stopped beating were selected as usual for the experiment. Twenty eggs were put into the solution of the following different salts, all in a concentration of M/32, and a control of M/32 sea water.

TABLE VII.

Influence of Anion upon Number of Hearts Recovering from KCl.

After	Sea water	NaCl	NaBr	NaI	$NaNO_3$	NaCNS	Na acetate	Na_3 tartrate	Na_2SO_4	Na_3 citrate
hrs.										
2¼	0	2	3	4	2	0	2	6	5	10
4¼	2	4	6	8	5	4	6	12	13	14
5¼	5	9	8	10	11	7	12	14	19	11
9¼	11	14	18	17	16	20	19	19	20	Dead.

The most important results are those obtained after two and three-quarters and four and one-quarter hours. They show that the relative efficiency of the anions follows the order from the most efficient to the least efficient:

Citrate > sulphate > tartrate > acetate, iodide > Br > Cl, NO_3.

The position of NaCNS is doubtful; it acts slowly, but all hearts finally recover. The sea water is less efficient even than NaCl, indicating an inhibiting effect of some substance contained in the sea water (Mg or NaOH?).

The case of Na_3 citrate requires a short discussion. It is obvious that at first the number of eggs which recover in Na_3 citrate is greater than in any of the other salts, but the superiority is only evident at first. This strange result finds its explanation

in the fact that while the citrate is the most efficient ion in causing the recovery it is at the same time very toxic and after about four hours gradually kills the embryos. If we select a lower concentration in which the toxicity of the citrate is naturally less the greater relative efficiency of the citrate for recovery comes out very clearly.

In the following experiment (Table VIII) the number of eggs which recovered in very weak solutions of NaCl, Na_2SO_4, and Na_3 citrate was ascertained. The eggs had been kept for three hours in 3/8 M KCl and those whose hearts had stopped beating were selected for the experiment. Controls were made in H_2O and normal (M/2) sea water. Only ten eggs were put into each solution.

TABLE VIII.

After	Number of eggs previously poisoned by KCl which recovered in										
	H_2O	M/2 sea water	NaCl			Na_2SO_4			Na_3 citrate		
			M/64	M/128	M/256	M/64	M/128	M/256	M/64	M/128	M/256
hrs. 2	1	8	2	1	1	5	3	4	8	7	6

The experiment shows that M/256 Na_3 citrate in spite of its toxicity is indeed more efficient than M/64 Na_2SO_4 and considerably more efficient than M/64 NaCl in accelerating the recovery of the eggs from KCl poisoning. The relative efficiency of the three salts increases therefore with the valency of the anion.

Experiments were made with phosphates to further test the idea concerning the influence of the valency of the anion. Na_3PO_4, Na_2HPO_4, and NaH_2PO_4 were used. In the alkaline (Na_3PO_4) solution the eggs were killed too rapidly to give any result. The di- and monosodium phosphates were extremely efficient; the monosodium phosphate on account of its acidity could only be used in concentrations below M/32. The eggs were put for three and a half hours into M/2 KCl and those whose hearts had stopped beating were used for the experiment. Controls were made with H_2O, sea water, and NaCl solution of various concentrations. Ten eggs were put into each solution.

It appears that in M/256 Na_2HPO_4 the recovery is as rapid as in M/8 NaCl. NaH_2PO_4 is apparently just as efficient as Na_2HPO_4

TABLE IX.

Recovery from KCl in NaCl and Phosphates.

Nature of solution	Number of eggs previously poisoned by KCl which recovered after								
	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	8 hrs.	20 hrs.	44 hrs.	7 days.
H_2O		0	0	0	0	0	2	5	3
Sea water	0	0	2	2	2	5	10		10
M/2 NaCl	0	0	0	1	1	6	10		10
M/4 NaCl		0	0	1	2	5	9	10	10
M/8 NaCl			0	1	1	4	9	10	10
M/64 NaCl				0	0	0	5	5	7
M/128 NaCl				0		1	3	4	7
M/256 NaCl						0	1	1	2
M/512 NaCl							2	5	5
M/32 Na_2HPO_4	1	5	7	7	9	10	10		
M/64 Na_2HPO_4	0	1	2	3	4	7	10		
M/128 Na_2HPO_4		1	1	2	4	6	9	10	
M/256 Na_2HPO_4		0	0	1	1	2	9	9	
M/512 Na_2HPO_4		0	0	0	0	0	2	6	
M/32 NaH_2PO_4	0	0	1	2	2	1	0		
M/64 NaH_2PO_4	0	3	5	5	7	3	0		
M/128 NaH_2PO_4		0	1	2	5	6	2	1	
M/256 NaH_2PO_4		0	0	1	3	3	8	8	
M/512 NaH_2PO_4				0	0	0	2	9	

except that the acid of the solution kills the eggs before the recovery is complete. The effects in the lower concentrations of NaH_2PO_4 (M/128 and M/256) and after a short exposure (four to eight hours) are fully as good as those in Na_2HPO_4.

If we try to express the effect of the increasing valency of the anion in figures our results show that approximately

$$\text{M/16 NaCl} = \text{M/64} \begin{matrix} \text{Na}_2\text{SO}_4 \\ \text{or} \\ \text{Na}_2 \text{ tartrate} \end{matrix} = \text{M/256} \begin{matrix} \text{Na}_3 \text{ citrate} \\ \text{or} \\ \text{Na}_2\text{HPO}_4. \end{matrix}$$

This approximates Hardy's rule that the ion effect should be an exponential function of the valency.

We feel justified in stating that the accelerating effect of salts upon the recovery of hearts previously poisoned with KCl is

an anion effect inasmuch as it increases with the valency of the anion apparently in agreement with Hardy's rule, and inasmuch as the acetate is much more efficient than the chloride.

V. THE INFLUENCE OF THE CATION.

In the earlier experiments on the antagonization of KCl by NaCl and Na_2SO_4 in the adult fish, Loeb and Wasteneys were inclined to ascribe this effect to a competition between the Na and K for a common colloidal anion. This idea was based on the fact that Na_2SO_4 seemed to be about twice as efficient as NaCl; while in the case of an anion effect the efficiency of Na_2SO_4 should have been greater than twice that of NaCl (according to Hardy's rule on the influence of valency upon the precipitating effect). The experiments on the adult fish were limited by the fact that the majority of Na salts (*e.g.*, Na_2 tartrate, Na_3 citrate, etc.) are so toxic that they could not be tested for their antagonistic effect upon KCl.

In the new experiments on the recovery of eggs poisoned with KCl these difficulties did not exist and Hardy's rule was confirmed.

That the greater efficiency of Na_2SO_4 is due to the anion and not to the fact that this salt contains twice the amount of Na (though not twice the amount of Na ions) as NaCl, could be proved also by comparing the effect of $MgCl_2$ and $MgSO_4$ upon the recovery of eggs previously poisoned with KCl. Such an experiment is reported in Table X. Eggs were put into M/4 KCl for twenty-four hours and those whose hearts had stopped beating were selected for a recovery experiment. Ten eggs were put into each of the following solutions, M/8 $MgCl_2$, M/8 $MgSO_4$, M/8 NaCl, M/8 Na_2SO_4, and H_2O. The number of beating hearts in each solution was ascertained at various intervals.

TABLE X.

After	Number of beating hearts of eggs previously poisoned with KCl in				
	H_2O	M/8 $MgCl_2$	M/8 $MgSO_4$	M/8 NaCl	M/8 Na_2SO_4
hrs.					
2	0	1	2	3	5
5	0	2	6	5	8
11	1	3	9	7	10

The experiments show that M/8 $MgSO_4$ is more than twice as efficient as M/8 $MgCl_2$ although the concentration of the cation is the same in both solutions. This leaves no doubt that the difference in efficiency must be ascribed to the anion. At the same time it is obvious that M/8 NaCl is more efficient than M/8 $MgCl_2$ although the latter solution contains twice as much Cl as the former. This suggests the possibility that Mg may inhibit the recovery of the eggs from potassium poisoning, while the anions favor the recovery.

This idea led to an investigation of the effect of different cations upon the recovery of eggs previously poisoned by KCl. Eggs were put for twenty-five hours into M/4 KCl and those whose hearts had stopped beating were selected for the experiment. They were then distributed into M/8 LiCl, NaCl, RbCl, CsCl, NH_4Cl, $MgCl_2$, $CaCl_2$, $SrCl_2$, and $BaCl_2$ solutions and the number of those recovered (*i.e.*, whose hearts were beating) was ascertained. Ten eggs were put into each solution.

TABLE XI.

After	Number of hearts previously poisoned with KCl beating in									
	H_2O	M/8 LiCl	M/8 NaCl	M/8 RbCl	M/8 CsCl	M/8 NH_4Cl	M/8 $MgCl_2$	M/8 $CaCl_2$	M/8 $SrCl_2$	M/8 $BaCl_2$
hrs.										
2	0	7	5	0	1	4	2	4	1	0
5	1	7	8	1	1	5	2	7	2	2
10	1	10	10	2	1	6	3	6	2	3
48	0	10	10	1	0	6	5	6	4	1

Those eggs which had not yet recovered were then put into sea water to find out whether the salt solution had killed them or whether it had only prevented their recovery. The latter was the case with those in H_2O, $MgCl_2$, $SrCl_2$, RbCl, and NH_4Cl, which recovered very rapidly in sea water. In $CaCl_2$ four had been killed by the $CaCl_2$ and the same was true with some in $BaCl_2$. Those in CsCl recovered only very slowly, which may indicate a superposition of a Cs effect over that of K. Making allowance for such complications, the results are intelligible on the assumption that the anions of the solution (in this experiment the Cl ions) are responsible for the recovery

of the eggs from KCl; and that the cations may have only a retarding effect. The latter is a minimum in the case of Li, is but slightly greater in the case of Na, and rises rapidly in the case of NH_4, Rb, and Cs. As far as the alkali earth metals are concerned, it is great in Mg, less in Ca, and is greater again in Sr and Ba. The writers make this statement about a possible retarding effect of the cations not without reluctance. We shall see in the next section that the hydrogen ion, though a cation, favors the recovery, while HO, though an anion, does not favor the recovery.

VI. THE RÔLE OF ACIDS AND BASES IN THE RECOVERY OF ANIMALS POISONED WITH KCl.

It seemed of importance to ascertain the influence of acids and bases upon the recovery of embryos poisoned with KCl. The investigation of this problem is restricted by the high toxicity of both acids and bases, which when they diffuse through the membrane of the egg soon kill the embryo. It is, therefore, necessary to work with low concentrations of these substances and only consider the effect during the first few hours before the acid or alkali has had time to kill the embryo. Under such conditions it was found that if a trace of acid is added to distilled water the embryos may recover from potassium poisoning while otherwise they will not.

In one experiment eggs were put into a M/4 KCl solution for thirteen hours. Those whose hearts had stopped beating were distributed into the following solutions. Each solution contained ten eggs. (Table XII.)

It is obvious that the addition of 0.1 cc. $\frac{N}{10}$ acetic acid to 50 cc. of distilled water accelerates the recovery of the eggs almost as much as if they had been put into normal sea water. It is, moreover, obvious that the embryos are very soon killed by the acid itself as is indicated by the coagulation of such embryos. The acid becomes more efficient for the recovery of the eggs poisoned with KCl the higher the concentration of the acid; but at the same time the eggs are killed more rapidly by the acid.

Since the point is of importance another experiment may be quoted. Eggs had been put into H_2O for twenty-four hours and only a few recovered. The others were distributed into various

TABLE XII.

Nature of solution	Number of eggs previously poisoned in KCl recovered after					
	1 hr.	2 hrs.	3 hrs.	5 hrs.	10 hrs.	22 hrs.
H_2O	0	0	0	1	2	2
Sea water	0	1	3	7	8	10
50 cc. H_2O + 0.1 cc. $\frac{N}{10}$ acetic acid	0	2	3	4	6	3 (7 killed by acid).
50 cc. H_2O + 0.2 cc. $\frac{N}{10}$ acetic acid	0	2	4	3	1 (5 killed by acid)	All killed by acid.
50 cc. H_2O + 0.3 cc. $\frac{N}{10}$ acetic acid	1	1	2	3	1 (9 killed by acid)	All killed by acid.
50 cc. H_2O + 0.4 cc. $\frac{N}{10}$ acetic acid	2	3	3	3 (3 killed)		

solutions and the number of recoveries is stated in Table XIII. Each solution contained ten eggs.

The accelerating influence of a trace of acid upon the recovery from KCl poisoning is unmistakable. Slight effects were also obtained with very weak HCl, and citric and tartaric acids.

All attempts to obtain similar effects with bases (NaOH, NH_4OH, Na_2CO_3, Na_3PO_4) were in vain.

It is also possible to slightly retard the poisoning of the embryos through the addition of a trace of acid to the KCl solution. These experiments are, however, not so striking, possibly on account of the disproportion between the concentration of KCl and acid.

TABLE XIII.

Nature of solution	Number of eggs which recovered from previous poisoning with KCl after					
	1 hr.	2 hrs.	3 hrs.	4 hrs.	6 hrs.	18 hrs.
H_2O (21 eggs)	0	0	2	2	2	3
M/8 sea water	0	1	4	5	7	10
50 cc. H_2O + 0.2 cc. $\frac{N}{10}$ acetic acid	1	3	5	5	7	0 (all killed by acid).
50 cc. H_2O + 0.4 cc. $\frac{N}{10}$ acetic acid	0	3	4	Killed by acid.		

VII. ON THE IMMUNIZATION OF THE EGGS AGAINST KCl BY DISTILLED WATER.

We now come to the description of a curious group of facts which we found in the course of these experiments. We had noticed that eggs treated for twenty-four hours with a very dilute solution of KCl, which did not stop their heart beat, were much more resistant to a KCl solution of higher concentration than eggs taken directly from normal sea water and put into a KCl solution of the same concentration. This observation (which might have suggested to a layman the possibility of an "adaptation" to the poison) induced us to try the effects of a previous treatment of the eggs with distilled water before they were put into M/2 KCl. It was found that embryos which had been kept over night in H_2O were more resistant to KCl than eggs which had been put directly from sea water into a KCl solution of the same concentration. Thus in one case seventy-three eggs were put directly from sea water into a M/2 KCl solution and the same was done with ninety-six eggs of the same lot which had been kept for about twelve hours in distilled water. After three hours all the hearts of the first lot stopped beating, while only twenty-two of the ninety-six eggs previously treated with H_2O had stopped beating. Finally the latter eggs also succumbed to the influence of KCl, but it required a considerably longer time. The H_2O had brought about a change in the egg which retarded the poisonous action of KCl upon the embryo. It may be stated again that the embryo of *Fundulus* develops as normally in H_2O as in sea water.

Systematic experiments were then made in which the eggs were put for different lengths of time into H_2O before they were put into the M/2 KCl solution to find out how the H_2O would delay the action of KCl upon the eggs. The eggs were all of the same age. Twenty eggs were put directly from sea water into M/2 KCl and the number of beating hearts was ascertained each hour. The next twenty eggs were put for one-quarter of an hour into H_2O before being put into the M/2 KCl solution, the next lot of eggs were kept for one-half hour in H_2O before being put into the M/2 KCl solution, and so on. Table XIV gives the result of such an experiment.

The result is most striking. When eggs are put directly from

TABLE XIV.

Influence of Previous Treatment of Eggs with H_2O upon the Rate of Poisoning by KCl.

Time during which eggs had been in H_2O before being put into M/2 KCl	Number of hearts beating in M/2 KCl after			
	1 hr.	2 hrs.	4 hrs.	24 hrs.
Not in H_2O	4	3	1	0
15 min	9	2	2	0
30 min	12	4	3	0
1 hr	15	5	3	0
2 hrs	15	10	7	0
4 hrs	16	10		0
6 hrs	20			
22 hrs	20	18	13	2

sea water into M/2 KCl in one hour 80 per cent have no more heart beat; when the eggs are put for only fifteen minutes into H_2O before being put into the M/2 KCl solution only 55 per cent of the hearts stop beating in one hour. When they are put for six hours into H_2O before being put into M/2 KCl no hearts stop beating in one hour. When the eggs are put for a day into distilled water before being put into M/2 KCl they show a still higher degree of resistance to KCl.

This resistance to KCl is reversed but slowly when the eggs are put back into sea water after the H_2O treatment. It was found that in the eggs of one set all the hearts stopped beating when the eggs had been in M/2 KCl for two hours. Eggs of the same set that had previously been put for forty-seven hours into distilled water had all (with the exception of one) beating hearts after they had been exposed to the M/2 KCl solution for two hours. A third group of the same eggs was put into H_2O for twenty-four hours, then into sea water for eighteen, and was then submitted to M/2 KCl (simultaneously with the two other sets). After two hours, six hearts of this lot were still beating.

VIII. THE INFLUENCE OF THE CONCENTRATION OF ELECTROLYTES UPON THE SUBSEQUENT EFFECT OF KCl.

It may be well to discuss briefly a theoretical point before we go further. When eggs were poisoned with KCl so that the hearts had stopped beating they did not as a rule recover when

put into distilled water. Since the recovery cannot take pla unless the KCl diffuses out of the egg we will assume that t eggs do not recover in distilled water from KCl poisoning becau the KCl cannot diffuse from the egg into the distilled water. Th assumption will also explain why eggs which have been put fo some time into H_2O will be poisoned much more slowly whe put afterwards into a M/2 KCl solution. If we assume that th immersion of the eggs in the distilled water causes the formatio of a layer of distilled water in the network of fibrils forming th egg membrane we can understand that such a layer of H_2O forms as efficient a barrier against the diffusion of KCl through the membrane into the egg, as does the H_2O for the diffusion of KCl from the egg previously poisoned with KCl when such an egg is put into distilled water.

Considerations of this kind led us to expect that when we put eggs for some time into different concentrations of a salt solution previously to putting them into M/2 KCl the eggs should be the more resistant to the KCl the lower the concentration of the salt in which they had previously been kept.

Twenty eggs were placed for eleven hours into each of the following solutions: M/2 and M/8 sea water, and H_2O. From here they were transferred into M/2 KCl and the rate at which they were poisoned was ascertained. Each solution contained twenty eggs. Table XV gives the number of embryos whose hearts were still beating.

TABLE XV.

Eggs 11 hrs. in	Number of hearts beating in M/2 KCl after			
	1 hr.	5 hrs.	13 hrs.	24 hrs.
H_2O	20	19	16	13
M/2 sea water	3	2	0	0
M/8 sea water	20	17	8	0

While the embryos transferred into M/2 KCl from M/2 sea water had practically all ceased to have a heart beat after one hour (only three of twenty had heart beats) the hearts of those from M/8 sea water and from H_2O were at that time all still beating. The immunity induced by H_2O was, naturally, of greater duration than that given by M/8 sea water.

An experiment with various low concentrations of NaCl confirms this result and shows that below M/8 solutions a further lowering of the concentration of NaCl has comparatively little influence. Eggs were kept for twelve and one-half hours in M/16, M/32, M/64 NaCl, and H_2O and then transferred into M/2 KCl. Twenty eggs were used in each solution. Table XVI gives the result.

TABLE XVI.

Eggs previously kept for 12½ hrs. in	Number of hearts beating in M/2 KCl after				
	1 hr.	2 hrs.	3½ hrs.	6 hrs.	30 hrs.
M/16 NaCl	18	10	5	1	0
M/32 NaCl	19	8	2	1	0
M/64 NaCl	19	12	6	2	0
H_2O	19	17	8	3	0
M/2 sea water	7	1	1	0	

While the eggs taken from sea water succumbed to the KCl in less than two hours those from H_2O had practically all beating hearts at that time (seventeen out of twenty). Those from M/16, M/32, and M/64 NaCl were about midway between those from sea water and from H_2O.

Eggs that had been kept in weak KCl solutions without succumbing to the KCl also showed the effect of the dilution, *i.e.*, a greater immunity to M/2 KCl.

IX. THE RELATIVE TOXICITY OF DIFFERENT POTASSIUM SALTS.

The experiments thus far mentioned indicate that anions retard the diffusion of potassium into the egg, and accelerate such a diffusion out of the egg, and that this effect increases with their valency of the anion and is greater for acetate than for Cl. We have compared the relative toxicity of equimolecular concentrations of KCl, K acetate, and K_2SO_4. We should expect that in regard to toxicity the order should be KCl > K acetate > K_2SO_4, provided that the anions have an inhibiting effect upon the diffusion into the egg. For such experiments concentrations of M/8 or above must be used since we shall see later that concentrations of KCl below M/8 are so little poisonous that they cannot

be used for obtaining an answer to our question. We give two series with M/8 and M/2 solutions of KCl, K acetate, and K_2SO_4. Twenty eggs of the same set were put into each solution and the number of embryos with beating hearts was determined after certain intervals. Table XVII gives the result.

TABLE XVII.*

In	Number of embryos with hearts beating after							
	½ hr.	1 hr.	2 hrs.	3 hrs.	5 hrs.	9 hrs.	25 hrs.	60 hrs
M/8 KCl.	15	11	8	8	8	7	7	1
M/8 K acetate	20	18	14	12	14	15	14	1
M/8 K_2 sulphate.....	18	14	12	12	12	13	12	8
M/2 KCl.	14	8	2	0	0	0	0	0
M/2 K acetate.	17	7	4	1	1	0		
M/2 K_2 sulphate.	15	7	0	0	0			

* It should be stated that the sensitiveness of eggs to KCl solutions of lower concentrations differs slightly for eggs of different age and possibly also of different females. Thus the eggs used in Tables I and II were more sensitive than those used in Table XVII.

If we compare the effect of M/8 K acetate with that of M/8 KCl we notice that K acetate is less toxic than KCl; and the same is true for K_2SO_4, although in the latter solution the concentration of K is twice as great as in KCl.

The same result appears in the M/2 solutions though not quite so strikingly. These experiments therefore conform with the facts put into evidence in the previous chapters of this paper showing that the anions inhibit the diffusion of K into the egg.

We have mentioned that the lower concentrations of K salts are very little poisonous for the embryo. This fact is very remarkable and deserves attention. In Table XVIII are given the results of an experiment on the relative toxicity of different concentrations of KCl.

Twenty eggs (twelve days old) were put into each of the following KCl solutions and the rate at which the hearts stopped beating was ascertained.[6]

[6] It is possible that the hearts of younger embryos are affected more quickly by lower concentrations than the hearts of older embryos.

TABLE XVIII.

In	Number of embryos with beating hearts after									
	½ hr.	1 hr.	1½ hrs.	12 hrs.	24 hrs.	48 hrs.	76 hrs.	105 hrs.	192 hrs.	288 hrs.
M/2 KCl.	9	2	0	0	0	0	0	0	0	0
M/4 KCl.	16	9	7	4	4	4	1	0		
M/8 KCl.	19	15	11	14	16	16	13	12	6	0
M/16 KCl. ...	20	15	15	16	16	15	14	12	3	0
M/32 KCl. ...	20	20	20	20	20	19	19	19	5	0
M/64 KCl. ...	20	19	19	19	20	20	20	18	5	0
M/128 KCl. ..	20	20	20	20	20	20	20	20	6	1

The striking fact is that a M/32 KCl solution or below is not toxic while a M/16 solution is but slightly toxic. A M/16 KCl solution has approximately three times as high a concentration of KCl as that in which this salt is contained in the sea water. Beginning with M/8 the toxicity rises rapidly. The explanation is not simple. We might make the assumption that, beginning with very low concentrations, the inhibiting effect of the anion increases at first very rapidly with the increasing concentration of the anion, but after that increases more slowly with increasing concentration.

There is another explanation possible, based on the fact that if eggs are treated with H_2O or with a weak solution of some salt they become more resistant to KCl. It may be that in a weak solution of KCl the dilution effect makes itself felt, inasmuch as inside the meshes of the fibrils of the membrane of the egg a layer of H_2O or of very dilute sea water is formed which acts as a barrier to the further diffusion of KCl into the egg.

X. THEORETICAL REMARKS.

We have seen in this paper that when an embryo is poisoned by a potassium salt it cannot recover in H_2O or a saccharose solution; but that it will recover when put into the solution of an electrolyte; and that the latter's efficiency increases with the valency of the anion. As far as we can see there are two ways in which these facts might be explained: first, by assuming that the recovery is due to the outside electrolytes diffusing into the egg and acting directly on the embryo or heart, whereby

the latter recovers from its standstill caused by potassium. The second possible explanation is based on the assumption that the recovery of the embryo whose heart has stopped beating depends on the diffusing of the KCl out of the egg into the surrounding solution. On the basis of this assumption we should be forced to conclude that the diffusion of KCl from the membrane of the egg into H_2O or a sugar solution is practically impossible and that the surrounding solution must contain a certain concentration of anions which may be the smaller the higher their valency in order to allow the potassium to diffuse out.

It can be shown that the latter assumption is more probable, since Na_3 citrate, Na_2HPO_4, and Na_2 tartrate, which favor the recovery of the poisoned egg of *Fundulus*, are so toxic for the fish after it has left the egg that they kill it very rapidly; and that they cannot be used for the antagonization of potassium effects on the adult fish, since they kill the latter in much smaller doses than those required for the recovery of the embryo. Hence the efficiency of these anions for the egg must be based on the fact that they do not diffuse into the egg. As a matter of fact, we had to mention, in the discussion of our experiments on citrates, that only the results of experiments with low concentrations of this salt and of short duration could be used for the egg, since after a little while the citrate entering into the egg killed the embryo. It is, therefore, not possible to assume that the great effect of the citrates upon the recovery of eggs previously poisoned with KCl is due to a direct action of these salts on the embryo.[7] A second argument in favor of the diffusion theory is the fact that if eggs are put for some time into H_2O before being put into the potassium solution, the latter will poison the egg much more slowly. This is only intelligible on

[7] In passing we might remark that the former experiments on antagonistic salt action in the embryo of *Fundulus* all indicated that the antagonistic action consisted in the prevention of the diffusion of the outside salt solution into the egg. Thus the *Fundulus* embryo, as long as it is inside the egg membrane, will keep alive and float in a solution of 50 cc. 3 M NaCl + 1 cc. 10/8 M $CaCl_2$ for five days, while the newly hatched fish is killed almost instantly in such a solution. A 10/8 M solution of NaCl + $CaCl_2$ is the upper limit in which the newly hatched fish can live. This topic has been sufficiently discussed in former papers to which the reader may be referred.

the basis of the assumption that the diffusion of KCl is retarded by the previous treatment with H_2O, since H_2O is not antagonistic to the toxic effects of KCl.

We are then driven to the conclusion that the action of electrolytes upon the prevention and the reversal of potassium poisoning in the embryo of *Fundulus* is due to an influence of these electrolytes upon the rate of diffusion of potassium through the membrane.

On this assumption our results would lead us to the conclusion that *the anions of the surrounding solution retard the diffusion of the K ions into the membrane (and the egg) and accelerate the diffusion of the K ions out of the membrane (and the egg)*. If the egg is poisoned with KCl and if it is put into H_2O or a saccharose solution, the egg cannot recover on account of the lack of anions in this solution. If normal eggs are put for some time into distilled water the latter enters into the meshes of the felt-like membrane. If such eggs are subsequently put into M/2 KCl the layer of distilled water inside the membrane acts as a barrier through which the progress of the diffusion of the K ions into the eggs is retarded; since the more peripheral fibers of the membrane containing potassium can no more give it off to a layer of distilled water inside the membrane than they can to distilled water on the outside. Finally, when normal eggs are put into a KCl solution the retarding influence of the anions of the K solution is increased by the influence of the anions of the electrolyte added, although this action is partly balanced by the cations of the salt added.

It is also of importance to point out that while the eggs poisoned with KCl can easily recover in LiCl or NaCl (and in the former more quickly than in the latter) they recover not at all or only slowly in solutions of RbCl or CsCl. It is also possible that bivalent cations directly inhibit the recovery since in NaCl the eggs recover more quickly than in $MgCl_2$ or $CaCl_2$.

In a former paper[8] one of us has already pointed out that the potassium behaves in these experiments very much like an invisible basic dye. When we stain the egg membrane with a basic dye, like neutral red, the membrane is readily decolorized when the stained eggs are afterwards put into salt solutions, while

[8] Loeb, *Proc. Nat. Acad. Sc.*, 1915, i, 473.

the stained eggs are not or are only very slowly decolorized when put into distilled water. If we try to stain eggs in a neutral red solution to which salt is added, we also notice a retardation of the staining. These facts suggest that the diffusion of KCl or of K through the membrane of the egg is a process which, in its initial stage at least, is analogous to the diffusion of a basic dye through the membrane. The common basis for both phenomena lies in the nature of the forces by which neutral red and K are held in the membrane. We may imagine that there exists in the membrane a colloidal anion to which the cation, like K or neutral red, is bound. These binding forces are counteracted by the anions of the surrounding solution.

The analogy between the behavior of potassium and neutral red shows itself also in the fact that when eggs stained with neutral red are put into an acid solution the stain is readily given off; and we have also seen that when the eggs are poisoned with potassium they give off the potassium readily in a faintly acid solution. This acid effect can be explained by assuming that the colloid of the membrane which binds the potassium or the neutral red cation is an amphoteric electrolyte, which forms a salt with the acid; this salt is comparatively strongly dissociated, the colloid becoming the cation. This colloidal cation is no longer able to bind the potassium or neutral red cation.

Another point requires discussion. It is much easier to demonstrate the accelerating influence of electrolytes upon the recovery of the heart (or the diffusion of KCl out of the egg) than the retarding influence of electrolytes upon the diffusion of KCl into the heart. The reason is probably this, that in the latter case the KCl must be present in rather high concentration in the outside solution while in the former case the concentration of KCl in the outside solution is almost zero, since it will contain only the traces of KCl which diffuse out of the egg.

It is also obvious that these experiments, in case they can be generalized, must lead us to a new conception of the mechanism of the diffusion of cations through membranes, inasmuch as the chemical or kindred forces which colloidal anions of the membrane exercise upon the diffusing cation seem to play a decisive rôle in the mechanism of diffusion. This has already been mentioned by one of us in a previous paper.

Donnan has developed the equations for the equilibrium of distribution of cations between diffusible and non-diffusible (colloidal) anions separated by an animal membrane. It is quite possible that his equations cover our results as far as cations are concerned. Besides the Donnan effect we have, however, a marked anion effect which is not covered by his theory.

XI. SUMMARY OF RESULTS.

1. It is shown in this paper that eggs of *Fundulus* poisoned with KCl are not able to recover when put into distilled water or a saccharose solution, while they will recover when put into a solution of a salt or when a trace of acid is added to the distilled water. Hearts which have not been able to recuperate when kept for days in solutions of non-electrolytes will recover quickly when put into salt solutions. The indicator for the potassium poisoning is the standstill of the heart of the embryo and for the recovery the resumption of the heart beats.

2. It is shown that the relative efficiency of the salts for inducing the recovery of the heart beat increases, first (within certain limits), with the concentration of the salt in the solution, and second, with the valency of the anion of the salt, the valency effect apparently following Hardy's rule.

3. One of us has already pointed out in a previous publication that this action of the egg towards potassium is somewhat analogous to its behavior towards neutral red. When *Fundulus* eggs, stained with neutral red, are put into distilled water they can not give off their stain; they give it off, however, when a trace of acid or some salt is added.

4. The behavior of both the basic dye and the potassium can be understood on the assumption that their diffusion presupposes their combination with a colloidal anion of the membrane. This combination is counteracted by the presence of an excess of anions, especially di- and trivalent ones, in the outside solution, and it is also counteracted by the presence of a trace of acid in the outside solution.

5. This action of the acid may be explained on the assumption that the colloid of the membrane which binds the potassium and the neutral red is an amphoteric electrolyte which through the

addition of the acid is transformed into a salt, in the dissoci
tion of which the colloid forms a cation which is no longer able
bind other cations.

6. It is shown that if eggs are previously treated with distille
water for some time the KCl requires a much longer time to brin
about the poisoning than if the eggs are put into the KCl solu
tion directly from sea water. This can be explained on th
assumption that by the immersion of the egg in distilled wate
traces of it will get into the network of fibrils constituting th
membrane and this layer of H_2O will act as a barrier blocking th
further diffusion of the potassium through the membrane a
effectively as did the distilled water surrounding the membra
in the experiment mentioned in 1.

THE MEASUREMENT OF TOXICITY.

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(Received for publication, September 3, 1915.)

The writer has had occasion to determine the toxicity of a variety of substances by measuring their effects on the electrical conductivity of living tissues. The advantage of this method is that it enables us to follow the reaction from moment to moment and admits of a fairly high degree of precision.

One striking result of these investigations is to emphasize the fact that the relative toxicity of two substances may depend very largely upon the stage of the reaction at which the measurement is made. This is evident from an inspection of the curves in Figure 1. These represent the electrical resistance[1] of tissues of the marine alga *Laminaria* in sea water and in two toxic solutions. The resistance of the tissue in the normal environment of sea water is taken as 100 per cent. If the tissue be placed in a solution of NaCl of the same conductivity as sea water the resistance falls, somewhat as shown in Curve A, until it reaches the death point at 10 per cent, after which there is no change in resistance. If, on the other hand, the tissue be placed in a solution of some substance, which (like $CaCl_2$, $LaCl_3$, etc.) causes a rise, followed by a fall in resistance, we may get a curve somewhat like that shown at B.

The most common method of determining the toxicity of a solution is to determine the time necessary to cause death. But it is evident from an inspection of the curves that it is impossible to determine the precise moment of death, since they approach

[1] The method of measurement is described in *Science*, 1912, xxxv, 112. For applications of the method see Osterhout, W. J. V., *Science*, 1912, xxxvi, 350; 1913, xxxvii, 111; 1914, xxxix, 544; 1914, xl, 488; 1915, xli, 255; *Jour. Biol. Chem.*, 1914, xix, 335, 493, 517; *Bot. Gaz.*, 1915, lix, 317, 464; *Jahrb. f. wiss. Bot.*, 1914, liv, 645.

the axis asymptotically. This is doubtless true of death in all cases. It is therefore obvious that the death point does not offer a perfectly satisfactory criterion of toxicity.

We may avoid this difficulty by taking as a criterion the time needed to reach any convenient point on the curve, as, for example

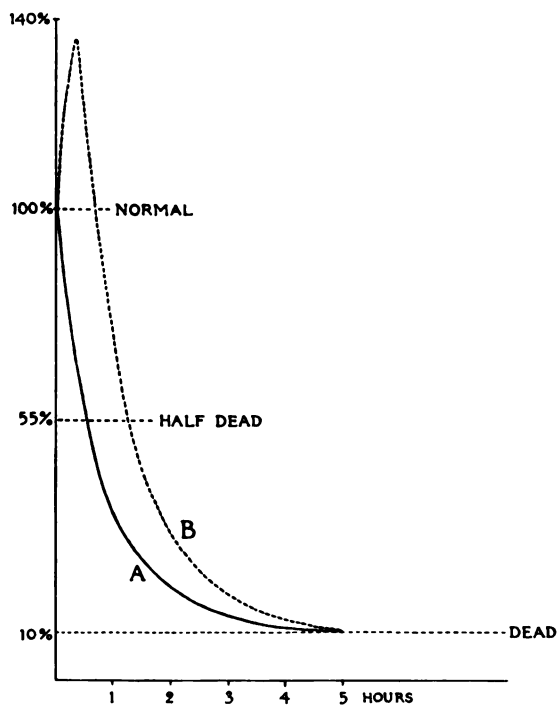

FIG. 1. Curves showing changes in the electrical resistance of tissues in two toxic solutions A and B (the latter causes a rise followed by a fall in resistance). Toxicity may be measured by determining the time required to carry the reaction to 55 per cent, which is half way between the normal condition and the death point.

55 per cent (half way between the normal condition and the death point). This may be determined with a good deal of precision by the measurement of electrical resistance or by any method which permits us to follow the reaction accurately from moment to moment. But where this cannot be done we may

employ other criteria. We may assume that as the reaction goes on certain phenomena appear at definite points on the curve, such, for example, as changes in metabolism, cessation of motion, or loss of irritability. The employment of such criteria may give trustworthy results in many cases if proper precautions are taken.

In the employment of any of these criteria, except that of death, we may meet the difficulty that the relative toxicity of two substances may vary greatly according to the point in the curve at which the comparison is made. Let us suppose that two toxic substances are so chosen that they produce death at about the same time, giving curves as shown in Figure 1. They must be regarded as equally toxic if we adopt death as the criterion but as unequally toxic if we take any other criterion. For example at 90 per cent A is seven times as toxic as B.

It is clear that we cannot escape from this difficulty by comparing the effects produced in equal times.

In view of these facts it is obviously undesirable to compare results obtained by the use of unlike criteria, as is often done.

The writer has found that the action of a number of toxic substances, as measured by the electrical method, follows the course of a monomolecular reaction.[2] In such cases the constants which express the reaction velocities of the two reactions afford the best measure of their relative toxicity.

In cases where such constants cannot be used but where the complete curve can be obtained the writer suggests the adoption, as an arbitrary standard, of the time necessary for the reaction to proceed half way to the death point. But when the curves are related to each other as are A and B in Figure 1 it may be desirable to use some other criterion. It is in any case desirable to give the whole curve whenever possible so that the reader may apply his own criterion.

The ease with which complete curves can be obtained by determining electrical resistance may render this method useful, especially since the writer has found it possible to apply it to all parts of plant tissues as well as to some animal tissues.

The electrical method is not restricted to solutions of the

[2] Osterhout, *Science*, 1914, xxxix, 544.

same conductivity. For example we find that NaCl 0.52 M and $CaCl_2$ 0.278 M have the same conductivity as sea water. If we wish to compare the toxicity of NaCl 0.278 M with that of $CaCl_2$ 0.278 M we may dilute the sea water until it has the conductivity of NaCl 0.278 M. Tissue placed in this may be used as a control. At the outset we make the resistance of the control equal to that of the tissue in NaCl 0.278 M or we divide the resistance of the control by a figure which reduces it to the same value (and divide all subsequent readings of the control by the same figure). We then express all readings of the tissue in NaCl 0.278 M as per cent of the reading of the control which is taken at the same time. All readings of the tissue in $CaCl_2$ 0.278 M are likewise expressed as percentage of the readings of a control in sea water having the same conductivity as $CaCl_2$ 0.278 M. Stronger solutions may be treated in the same way, using sea water which has been concentrated by evaporation.

Attention may be called to a further difficulty in determining toxicity. If tissue of *Laminaria* be transferred from sea water to pure solutions of toxic salts their relative toxicity sometimes appears to be different from that which is observed when the same substances are added directly to the sea water. Similar considerations may be found to apply to animals and plants which live on land or in fresh water, in which cases Ringer's solution or the water of soils and rivers may play the same rôle as the sea water in experiments with marine forms.

It may be added that in some cases variations in the supply of oxygen may cause changes in relative toxicity; and in view of the fact that the temperature coefficient is not the same in all cases of toxic action it seems desirable to carry out determinations as far as possible at a standard temperature, preferably at 18°C.

SYNTHESIS OF NORMAL TRIDECYLIC AND TETRACOSANIC ACIDS.

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(Received for publication, September 27, 1915.)

In a previous communication[1] Levene and West reported the results of a renewed investigation into the melting points of several of the higher normal fatty acids. The change in the melting points of the acids with the increase of the number of carbon atoms in the chain was found consistent with the generally accepted rule. Only tridecylic acid showed a much higher melting point than would be expected. It was mentioned in that article that it was intended to make this discrepancy the subject of a separate investigation.

In the previous work tridecylic acid was prepared by oxidation of α-hydroxy-myristic acid. It is evident that the acid prepared by this method may contain traces of myristic acid, if there was exercised a lack of care in the purification of the α-bromo- or α-hydroxy-myristic acids. It was, therefore, concluded to synthesize the acid by the malonic ester method.

The acid obtained in this matter had a melting point of 44.5–45.5° (corrected). In order to secure greater certainty regarding this finding on the tridecylic acid obtained through the malonic ester synthesis the substance was prepared independently by two of the present authors. The physical properties of the two substances were identical.

It was then concluded to repeat the preparation of the tridecylic acid by the oxidation of α-hydroxy-myristic acid. In this instance care was taken to remove completely traces of myristic acid. The acid prepared from the pure hydroxy-myristic acid

[1] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1914, xviii, 463.

had the same melting point as that obtained through malonic ester.

The tetracosanic acid was prepared because of the bearing the acid had on the structure of lignoceric acid. Meyer, Brod, and Soyka[2] were the first to express the view that lignoceric acid differed in structure from the normal tetracosanic acid. They prepared the latter synthetically and determined its melting point. According to them the melting point exceeded that of behenic acid only by 1.5–2°. Since the authors failed to isolate the intermediate product, there always remained some doubt as to the absolute purity of their product.

In the present investigation the twice recrystallized docosylmalonic acid served for the preparation of tetracosanic acid. Recrystallized from toluene the substance had the melting point of 87.5–88°. The acid prepared by Meyer, Brod, and Soyka melted at 85.5–86°.

In this work the melting points were determined in a sulphuric acid bath provided with a stirrer; the rate of heating was uniform—7 to 8 seconds for each degree.

EXPERIMENTAL PART.

Tridecylic Acid.

Undecylic Alcohol,[3] $C_{11}H_{23}OH$.—Undecylic alcohol was prepared by reducing 50 grams of ethyl undecylate with 35 grams of absolute ethyl alcohol and 32 grams of metallic sodium. The yield was 23 grams of alcohol, which boiled at 147° at 25 mm. pressure.

Undecylic Iodide, $C_{11}H_{23}I$.—60 grams of undecylic alcohol, 45 grams of iodine, and 8 grams of red phosphorus were heated one and one-half hours at 180°. The reaction product was taken up in ether, the solution shaken with aqueous sodium thiosulphate, and dried. 85 grams of iodide were obtained, boiling at 125° at 3 mm. pressure, and at 117° at 1.3 mm. pressure.

0.1663 gm. of iodide gave 0.1378 gm. AgI (Carius).

	Calculated for $C_{11}H_{23}I$:	Found:
I	44.99	44.79

[2] Meyer, H., Brod, L., and Soyka, W., *Monatsh. f. Chem.*, 1913, xxxiv, 1133.

[3] Jeffreys, E., *Am. Chem. Jour.*, 1899, xxii, 37.

Ethyl Undecylmalonate, $C_{11}H_{23}CH(COOC_2H_5)_2$.—The condensation was carried out as follows: 2.05 grams of sodium were dissolved in 50 cc. of absolute ethyl alcohol, 14.2 grams of ethyl malonate and 25 grams of undecylic iodide were added, and the mixture was heated three hours on the water bath under a reflux. The reaction product was treated with water, the ester extracted with ether, the ether solution washed with water and dried. Ethyl undecylmalonate boils at 208–209° (corrected) at 21 mm. pressure.

0.3506 mg. of ester required 21.0 cc. $\frac{N}{10}$ NaOH for saponification. Calculated, 22.4 cc.

Undecylmalonic Acid, $C_{11}H_{23}CH(CO_2H)_2$.—For the preparation of the dibasic acid it is unnecessary to distill the ester. The crude product, obtained upon concentrating the ether solution, was saponified by warming with an excess of 50 per cent sodium hydroxide. The soap was washed twice with dry acetone and then decomposed with concentrated hydrochloric acid. The free acid was extracted, taken up in acetone, the solution filtered, the acetone removed on the steam bath, the product washed with petroleum ether and crystallized from benzene and then from a mixture of acetone and petroleum ether. It melts at 108.5° (corrected) without decomposition.

0.300 gm. of substance, dissolved in a mixture of ethyl alcohol and benzene, required 22.78 cc. $\frac{N}{10}$ NaOH for neutralization.

	Calculated for $C_{14}H_{28}O_4$:	Found:
Mol. Wt.	258.2	263.4

Tridecylic Acid, $C_{12}H_{25}COOH$.—The dibasic acid was heated at 180° for one hour or until the evolution of carbon dioxide had practically ceased. The resulting tridecylic acid was then distilled over in vacuum. Two fractions were collected, the first boiling between 148° and 153° at 1.2 mm. pressure, the second, between 153° and 158° at the same pressure. A second experiment gave a product boiling at 199–200° at 24 mm. pressure (corrected). Each fraction was twice crystallized out of dry acetone. The tridecylic acid thus obtained melted at 44.5–45.5° (corrected).

0.500 gm. of substance, as above, neutralized 23.4 cc. $\frac{N}{10}$ NaOH.

	Calculated for $C_{13}H_{26}O_2$:	Found:
Mol. Wt.	214.2	213.7

In order to determine whether the method or the material was at fault in our earlier work, we repeated the preparation of tridecylic acid by the oxidation of α-hydroxy-myristic acid with potassium permanganate. The acid thus obtained was identical with the synthetic product from undecylic acid.

1.00 gm. of acid, as above, neutralized 47 cc. $\frac{N}{10}$ NaOH.

	Calculated:	Found:
Mol. Wt.	214.2	212.8

This indicates that the method is satisfactory, and shows that with pure hydroxy acids pure monobasic acids may be obtained.

Tetracosanic Acid, $C_{23}H_{47}COOH$.

Ethyl Docosylmalonate, $C_{22}H_{45}CH(COOC_2H_5)_2$.—0.53 gram of sodium was dissolved in 50 cc. of absolute ethyl alcohol and to the cooled solution 3.68 grams of ethyl malonate and 10 grams of docosyl iodide, $C_{22}H_{45}I$, were added. The mixture was heated on the water bath for twenty-four hours. The reaction product was diluted with water. The ester which separated out was washed with water and recrystallized twice from acetone. The yield was about 10 grams. The analyzed product was obtained by esterifying a sample of docosylmalonic acid which had been twice crystallized out of acetone. It melts at 48° (corrected).

0.1026 gm. of substance gave 0.2798 gm. CO_2 and 0.1138 gm. H_2O.

	Calculated for $C_{29}H_{56}O_4$:	Found:
C.	74.29	74.38
H.	12.08	12.45

Docosylmalonic Acid, $C_{22}H_{45}CH(CO_2H)_2$.—The above ester was dissolved in boiling alcohol and about five equivalents of 50 per cent sodium hydroxide were added, and the mixture was boiled. The soap which separated out upon cooling the alcohol was filtered off, washed with water, and then extracted thoroughly with boiling acetone. The free acid was liberated by treating

the soap with concentrated hydrochloric acid. This was purified by recrystallizing it from acetone. Various methods were tried to obtain a product which would give a correct molecular weight. As this was not readily accomplished, due to the difficulty of removing the last traces of alkali, the dibasic acid was changed into the monobasic after two recrystallizations from acetone.

Ethyl Tetracosanate, $C_{23}H_{47}CO_2C_2H_5$.—Docosylmalonic acid was heated one hour at 160–180° and the crude reaction product esterified by boiling with 5 per cent sulphuric acid. After repeating the esterification three times the ester was recrystallized twice out of acetone, distilled in vacuum, again recrystallized, distilled, and recrystallized. The product then boiled at 118° at 0.6 mm. pressure and melted at 56–57° (corrected).

0.100 gm. of substance gave 0.2893 gm. CO_2 and 0.1173 gm. H_2O.

	Calculated for $C_{26}H_{52}O_2$:	Found:
C	78.79	78.90
H	13.13	13.15

Tetracosanic Acid, $C_{24}H_{48}O_2$, $C_{23}H_{47}COOH$.—The ethyl ester was dissolved in boiling alcohol, and five times the equivalent of sodium hydroxide added for saponification. The soap was washed with water and thoroughly extracted with boiling acetone. The acid, which was liberated by boiling with concentrated hydrochloric acid, was purified through the lead salt. From toluene, tetracosanic acid crystallizes in scales, melting at 87.5–88°. Meyer gives 85.5–86°.

0.400 gm. of acid neutralized 10.9 cc. $\frac{N}{10}$ NaOH.

	Calculated for $C_{24}H_{48}O_2$:	Found:
Mol. Wt.	368.5	367

THE METABOLIC RELATIONSHIP OF THE PROTEINS TO GLUCOSE.

III. GLUCOSE FORMATION FROM HUMAN PROTEINS.

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(Received for publication, September 9, 1915.)

It is known that the proteins of the human body contribute to form glucose in severe cases of diabetes mellitus, but considerable doubt exists regarding the exact extent of this sugar formation. Thus it has been variously estimated that from 45 to even 80 per cent of body protein is convertible into glucose.[1] These calculations have usually been based on the relative amounts of glucose and nitrogen excreted in the urine of diabetic human subjects or animals.

In such diabetes mellitus experiments the results are nearly always of doubtful value, for aside from dietary considerations, there is a lack of conclusive data showing that the power of the human diabetic organism to utilize glucose is completely extinguished. This criticism does not apply, however, to fully developed phlorhizin diabetes in the human subject, which has been observed by Benedict and Lewis to show a urinary G : N ratio of 3.6 : 1 on a carbohydrate-free diet. Only a single case was studied. About the same value has been reported for severe diabetes mellitus.[2] But the ratio in which glucose appears in relation to nitrogen in the urine is unfortunately not in all cases a certain basis for calculation of the maximal amount of sugar capable of being formed by the protein of the organism.

[1] Noorden, C. H. v., Die Zuckerkrankheit und ihre Behandlung, Berlin, 6th edition, 1912, 11. Falta, W., Die Erkrankungen der Blutdrüsen, Berlin, 1913, 438.

[2] For literature and statements in the text requiring further explanation see the immediately preceding article of this series, Janney, N. W., and Csonka, F. A., *Jour. Biol. Chem.*, 1915, xxii, 203.

When, however, protein either isolated or in the form of fre muscle is fed to fasting phlorhizinized dogs under observance proper precautions, glucose originating in the metabolism of t protein may be determined with considerable exactness. Maki use of the same experimental conditions and methods alrea described in previous communications, *human* muscle was accor ingly fed to phlorhizinized dogs and the sugar formed from t proteins contained in this material calculated in the manr hitherto employed.

As an average result of five experiments it was found that 5 per cent of the muscle protein had been converted into gluco The average protein G : N ratio obtained by dividing this val by the amount of nitrogen contained in 100 grams of protein 3.53 : 1. If from the protein nitrogen 0.34 gram be deducted nucleic acid which in all probability yields no glucose, *the ra 3.60 : 1 may be accepted as representing the relation between the nit gen contained in human muscle protein and the glucose originati from the same in metabolism.* The muscle proteins constitute t chief bulk of body protein. The average amount of metabo glucose yielded by other body proteins (serum albumin, gelat fibrin) is 57.7 per cent. *It may on these grounds be properly c cluded that the proteins of the human organism collectively may yi a maximum of about 58 per cent of glucose in diabetic metabolis*

With regard to this mode of experimentation the question m arise whether the glucose formation from muscle fed in such d experiments can be accepted as the same which would occ from the proteins of the fasting, completely diabetic human ganism. It is believed, however, that results previously obtain justify the acceptance of this view. It was shown that the g cose formation from ingested body protein (dog muscle and i lated dog muscle protein) closely corresponds to the amount sugar formed from the proteins of the living, fasting, complet diabetic canine, which has been most carefully studied.

The following may also be added. Body proteins of t different vertebrates examined all show the same general compo tion and yield about the same relative amount of metabolic g cose. Approximately the same values can also be calculat from the glucogenetic amino-acids contained in such protei It may therefore be concluded that human proteins would be fou

likewise to yield about the same amount of glucose in metabolism. This is indeed the case. In the following table these values for man, dog, and rabbit from direct feeding experiments as described, are compared to similar values calculated for the ox and chicken by using data afforded by amino-acid determinations (see preceding articles).

Glucose Yielded in Metabolism of Proteins of Higher Animals.

Species.	Man.	Dog.	Rabbit.	Ox.	Chicken.
Protein G: N ratio..	3.6: 1	3.6: 1	3.8: 1	3.6: 1	3.4: 1
Glucose per 100 gm. of protein.....	58	57.5	60	57.5	54.5

The absence of a sufficient number of urinary examinations made in fasting cases of severe human diabetes renders it impossible to establish with certainty an average *urinary* G: N ratio for fully developed diabetes mellitus as in the case of starving phlorhizinized dogs. This ratio can, however, be calculated from the protein G: N ratio 3.60 : 1 for man. In such a calculation it must be remembered that creatine, creatinine, and purine nitrogen, estimated as 5.5 per cent for the human species, must be left out of account, as these substances are non-glucogenetic. *In the fasting human diabetic of the severest type, when glycogen and possible sources of glucose other than protein are exhausted, the urinary G : N ratio of 3.4 : 1 may therefore be accepted as an average value.* The same value has been established for the fasting phlorhizin diabetic dog.

The urinary G :N quotient of man seems to be capable of clinical application, as Lusk[3] has previously suggested. When a lower G: N ratio prevails during a protracted fast, now the most modern treatment for diabetes,[4] it may be reasonably inferred that the organism has not yet entirely lost its power to utilize glucose. The prognosis is therefore more favorable. Conversely when the G : N ratio ranges about 3.4 : 1 a grave prognosis

[3] Mandel, A. R., and Lusk, G., *Jour. Am. Med. Assn.*, 1904, xliii, 241.
[4] Allen, F. M., *Boston Med. and Surg. Jour.*, 1915, clxxii, 241.

may be made, as all the glucose capable of being formed from protein is being lost to the body.

From the above it is evident that not more than about 60 per cent of body protein, corresponding to a urinary G: N ratio of about 3.4 : 1, can be converted into glucose. The statements in the literature of much higher ratios, such as 5 : 1, and a correspondingly greater sugar formation from the proteins of the organism, can therefore be no longer accepted.

Analysis of Human Muscle Used in Feeding Experiments. Grams per 100 Grams.

	Total solids.	Nitrogen.	Reducing substances.	Glycogen.	Total protein.	Nitrogen per 100 gm. protein.
Human Muscle I (first two experiments).....	19.35	2.35	0.12	0.13	12.22	16.36
Human Muscle II (last three experiments).....	12.57	1.92	0.20	0.19	9.11	16.48

Human Muscle Feeding Experiments.

Muscle fed.	Protein fed.	Nitrogen fed.	Weight of dog.	Periods.	Nitrogen.	Glucose.	G : N.	Extra glucose.	
								Amount.	In terms of protein fed.
gm.	*gm.*	*gm.*	*kg.*	*hrs.*	*gm.*	*gm.*		*gm.*	*per cent*
				24	10.94	40.61	3.71		
165.1	20.18	3.88	12.7	24	12.50	42.73	3.42	12.10	59.96
				12	5.70	19.80	3.48		
				24	11.24	36.59	3.26		
166.2	20.31	3.90	12.8	24	12.80	40.27	3.15	11.48	56.52
				12	5.40	16.33	3.02		
				12	4.99	15.94	3.20		
				12	6.35	19.90	3.13		
242.2	22.07	4.65	15.2	24	14.51	45.46	3.06	13.01	58.95
				24	12.67	40.82	3.22		
235.8	21.48	4.52	14.8	24	13.87	43.24	3.12	11.35	52.84
				24	11.92	40.54	3.40		
216.7	19.74	4.16	13.6	24	11.71	39.22	3.35	12.23	61.96
				24	9.76	34.29	3.51		

THE DETERMINATION OF REDUCING SUGARS.

A VOLUMETRIC METHOD FOR DETERMINING CUPROUS OXIDE WITHOUT REMOVAL FROM FEHLING'S SOLUTION.[1]

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INTRODUCTION.

In the course of an investigation, still in progress, of the physiological activity of a group of soil organisms, it became necessary to determine the quantity of dextrose in a series of solutions. It was desirable to have a method as accurate as the Fehling gravimetric one but which would yield results about as quickly as by direct titration. The reducing sugar present in the 150 cc. of each of these solutions varied from nearly zero to about 550 mg. A definite quantity of a copper salt had to be added to each solution. Direct titration could not be used unless this salt was first removed and the solutions of small sugar content were concentrated, a procedure which would make this method a very long one. There were no such difficulties if the gravimetric method was employed, as less copper sulphate solution could be used in mixing the Fehling's reagents and the sugar solution could then be added directly.

The new volumetric method was devised for the determination of the cuprous oxide in the Fehling's solution. It consists in converting the oxide into cuprous chloride and then pipetting a definite volume into a known quantity of iodine solution. The iodine which is not reduced is titrated with sodium thiosulphate solution.

Since this method was completed the author's attention has been called to the work of Rupp and Lehmann[2] who also de-

[1] Published by the permission of the Secretary of Agriculture.

[2] Rupp, E., and Lehmann, F., Ueber die K. Lehmann'sche Titration von Zuckerarten, *Arch. d. Pharm.*, 1909, ccxlvii, 516.

scribe a procedure for determining cuprous oxide in Fehling's solution. Their method is entirely different in principle and is not nearly as accurate[3] as the proposed one. Among other faults it necessitates the use of the whole Fehling's solution so that duplicate titrations cannot be made.

The time required for a single determination by the new method is not over twenty minutes; that is, it takes ten minutes more than the time required for the whole Munson and Walker procedure up to the filtration of the cuprous oxide. When a series of determinations is to be made, the technique can be modified so that the time required for a single one will be much less.

When dextrose, maltose, lactose, or invert sugar are oxidized by Fehling's solution, the acids formed are without action on iodine, so that these sugars and no doubt the other reducing ones may also be determined by this method.

Method.

Apparatus.—While no special apparatus is necessary for this method a slight modification is made in the usual form of a 200 cc. volumetric flask. This flask, marked to contain, is cut off 20 mm. above the mark. The edge is polished and flared a little so that a rubber stopper may be inserted and pressed down to within 12 or 14 mm. of the capacity mark.

As it is not always convenient to time a pipette for the delivery of a definite volume the author has substituted the method of calibrating a 50 cc. pipette against the 200 cc. volumetric flask and then placing a small file mark near the tip of the pipette at the point where the solution stops. Four times the volume contained between the mark on the stem and the tip of the pipette should exactly fill the flask.

A rubber stopper, with one hole, that will fit a 500 cc. Erlenmeyer flask is slipped over the tip of the 50 cc. pipette into position on the stem so that when the stopper is in the flask the tip of the pipette will be about 1 cm. below the surface of the 270 cc. of solution which the flask contains. A small V-shaped vent should be cut in the side of the stopper. As two flasks are used

[3] In the determination of 24.2, 27.3, 48.6, and 117. mg. of dextrose the error was 2., 2.9, 1.4, and 0.8 per cent respectively.

it is well to pick out two with mouths of about the same diameter. Rubber stoppers without vents are placed in each of these flasks.

A 25 cc. volumetric pipette is also marked on the tip at the point where the solution stops, so that exactly the same volume may be measured each time; the absolute volume in this case is of no importance.

Procedure.—The procedure for making a determination is comparatively simple and lends itself well to routine work.

The Fehling's solutions[4] are mixed in a 400 cc. Jena beaker, in the proportions recommended by Munson and Walker,[5] the sugar solution is poured in, and the volume made up to 100 cc. with distilled water. The beaker is covered with a watch glass and placed on an asbestos mat over a Bunsen burner with the flame so regulated that boiling begins in four minutes. The boiling is continued for two minutes.

As soon as the Fehling's solution is placed over the flame, approximately 250 cc. of distilled water are poured into each of the 500 cc. Erlenmeyer flasks. 25 cc. of iodine[6] are then pipetted into each flask. The iodine must be very accurately measured so that exactly the same volume that was used for the standardization will be obtained. The 25 cc. pipette with the mark on the tip acts as a check to prevent any mistakes in this desired accuracy. When the iodine is in the flasks they are sealed with a rubber stopper.

A 75 mm. funnel with a long stem is placed in the 200 cc. volumetric flask and then 15 cc. of concentrated HCl are poured into it.

Approximately 15 cc. of water (between 80° and 95°) are added to the acid in the volumetric flask just before the boiling of the Fehling's solution is completed. The tip of the funnel should be below the surface of this solution.

[4] The modified Fehling's solutions recommended by Soxhlet were used. Solution A contains 34.639 gm. of $CuSO_4 \cdot 5H_2O$ in 500 cc. of water. Solution B contains 173 gm. of Rochelle salts and 50 gm. of NaOH in 500 cc. of water.

[5] Munson, L. S., and Walker, P. H., Unification of Methods for Determining Reducing Sugars, *Jour. Am. Chem. Soc.*, 1906, xxviii, 663. Walker, P. H., *ibid.*, 1907, xxix, 541; 1912, xxxiv, 202.

[6] The $\frac{N}{10}$ iodine solution is prepared by dissolving 6.5 gm. of resublimed I and 9 gm. of KI in 1 liter of distilled water.

When the Fehling's solution has boiled two minutes the beaker is removed from the flame, the solution agitated to bring the precipitate into suspension, and then rapidly poured onto the funnel. The beaker, watch glass, and funnel are washed with hot water and the volumetric flask is then filled almost to the mark. A few cc. of water from the wash bottle are run into the beaker and then by means of a 1 cc. pipette the solution in the flask is brought up to the mark. The rubber stopper is immediately inserted and the flask covered with a towel while it is tilted back and forth five or six times or sufficiently to give the contents an even distribution. As a slight pressure develops in the flask it is well to place the finger over the stopper to prevent its being forced out. When the acid solution is satisfactorily mixed the stopper is withdrawn and a 50 cc. quantity of the solution immediately pipetted into each of the flasks containing iodine solution. The stem of the pipette is placed in the flask and the rubber stopper pressed down so that the pipette is held steady and its contents are delivered about 1 cm. below the surface of the solution. The Erlenmeyer flask is agitated occasionally to distribute the solution and prevent the formation of a precipitate by an excess of the acid solution accumulating in one place. As this solution drains down the rubber stopper is drawn out and the pipette tilted so that its contents will run out more slowly. The pipette is withdrawn as soon as the solution in it reaches the mark on the tip. If an attempt is made to stop the flow of the solution as it nears the mark by placing the finger over the end, in the usual way, some of the solution will frequently be drawn up from the tip by the contraction of the air in the pipette. Simple tilting of the pipette and the flask gives sufficient control to stop the flow when it reaches the mark. As soon as the pipette is empty it is removed and the flask sealed with a rubber stopper.

Where only one pipette is used to transfer the cuprous chloride solution to the iodine solution, three pipettefuls may be withdrawn without any error being introduced by the change in temperature of the solution, provided the pipette is again filled as soon as it is empty. In cool weather when a change may occur more quickly it may be necessary to use two pipettes so that the second 50 cc. quantity can be measured without waiting till the first pipette is empty or one pipette may be used and the volu-

metric flask placed in a large beaker containing water at about the temperature of the acid solution. The volumetric flask should be sealed with a rubber stopper to prevent the access of oxygen when the solution is not being taken from it.

The iodine remaining after the addition of the cuprous chloride solution is titrated with $\frac{N}{10}$ sodium thiosulphate,[7] 2 cc. of starch solution being added when the end-point is nearly reached. The solution at the end of the reaction is a very light green, but it is so light and different from the iodo-starch blue that it does not interfere with an accurate determination of the end-point.

The difference between the number of cc. of $\frac{N}{10}$ thiosulphate solution oxidized by 25 cc. of iodine solution, as determined by the standardization, and the number of cc. of $\frac{N}{10}$ thiosulphate acted upon by the iodine remaining after the addition of the cuprous chloride solution, is multiplied by 14.315 to obtain the mg. of Cu_2O in the whole solution. The constant 14.315 is equal to four times the number of mg. of Cu_2O that would be oxidized by 1 cc. of $\frac{N}{10}$ iodine solution.

Amounts of dextrose from 4 mg. up to 150 mg. may be determined with these $\frac{N}{10}$ solutions, but of course by either changing the strength of the reagents or taking a smaller quantity of the cuprous chloride solution this method can be made to cover the determination of reducing sugar up to the limit of the Fehling method.

Data.

In order to test the method a sample of pure dextrose was obtained from the Bureau of Standards[8] and a 0.5 per cent solution prepared. No copper salt was added to the sugar solution for these tests because they were made to determine the average accuracy of the method. These experiments, selected at random, will illustrate this accuracy.

[7] Standardized against $\frac{N}{10}$ $K_2Cr_2O_7$ solution.

[8] Bureau of Standards, Dextrose Sample No. 41; B. S. Test No. 16741-30.

Temperature of water.	$\frac{N}{10}$ thiosulphate solution = 25 cc. of iodine solution.	$\frac{N}{10}$ thiosulphate solution used to titrate excess iodine.		Dextrose present.	Dextrose found.	Error.
		Duplicates.	Average.			
°C.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*	*per cent*
87	24.90	24.04 24.08	24.06	5.0	4.91	−2.0
90	24.98	23.32 23.32	23.32	10.0	9.85	−1.5
90	24.04	19.78 19.78	19.78	25.0	25.08	+0.32
95	24.04	16.02 16.03	16.03	50.0	49.86	−0.28
90	24.98	9.37 9.41	9.39	100.0	99.97	−0.03
95	24.80	2.00 2.02	2.01	150.0	150.32	+0.21
97	24.80	1.99 2.01	2.00	150.0	150.39	+0.26

DISCUSSION.

The method described is one that may be used for the determination of widely varying quantities of reducing sugar, but if uniformly smaller quantities were to be determined the accuracy of the method may be increased by taking more of the cuprous chloride solution and using weaker titrating solutions. The method as outlined, however, is as accurate as the gravimetric one of Munson and Walker. All the figures in their table for less than 20 mg. of dextrose were obtained by interpolation, so there is no standard with which to compare the results obtained in the determination of the smaller quantities of dextrose. In working with this quantity of dextrose Munson's average result was 1 per cent lower and that of Walker 1 per cent higher than the amount of reducing sugar added.

As the results obtained in the determination of larger quantities of dextrose were as close to the theoretical values as those obtained with the gravimetric method it is evident that this volumetric method when properly manipulated will yield results fully as accurate as those of our standard gravimetric method.

If a blank Fehling's solution is put through this procedure it is found that from 0.1 to 0.2 cc. of iodine has been reduced. It is believed, however, that when cuprous chloride is entering the iodine solution this quantity of iodine, which may be changed by the heat of the solution entering, is not without action on the cuprous chloride; because by taking the 25 cc. of iodine solution in distilled water as the quantity which is acted upon by the cuprous chloride the correct amount of cuprous oxide is obtained. If the iodine is lost, this way of calculating the result is only equivalent to adding a constant; so no error is introduced.

In these experiments no precipitation of cuprous oxide was obtained by boiling the blank Fehling's solution. If there should be a precipitate of 0.3 or 0.4 mg. of cuprous oxide it could not be determined with these reagents, as one-fourth of it or 0.1 mg. would be equal to 0.03 cc. of the $\frac{N}{10}$ solution and this value would of course be covered by experimental error.

This method may be used for the quantitative determination of copper by reducing the metal in an alkaline solution with dextrose and then following the procedure.

SUMMARY.

A new volumetric method is described for the determination of cuprous oxide in Fehling's solution. The cuprous oxide without filtration from the solution is converted into cuprous chloride and then pipetted into a known quantity of a dilute iodine solution. The iodine which is not reduced is then titrated with sodium thiosulphate solution.

The data indicate that the method will give results as accurate as those obtained with the gravimetric method.

Dextrose, maltose, lactose, invert sugar, and no doubt other reducing sugars may be estimated by this procedure.

This method yields results in one-quarter of the time required to obtain them by the gravimetric method.

The method may also be used for the quantitative determination of copper.

IE ACID-BASE EQUILIBRIA IN THE BLOOD AFTER PARATHYROIDECTOMY.

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(Received for publication, August 28, 1915.)

The tetany which develops after the removal of the parathyroid ands has been the subject of many investigations attempting to certain the cause or to determine methods of relief. While the ecific cause for the tetany may hardly be considered satisfactorily emonstrated, a number of procedures have been found which use temporary relief during which the gross symptoms of cessive muscular stimulation are either partially or totally ssipated.

The relief from parathyroid tetany observed after the administation of acids as shown by some of us in a recent communication[1] ggests the possibility of a peculiar abnormality of metabolism ereby the normal equilibrium of acids and bases in the body disturbed, resulting in a relative increase in the amount of se. This condition may quite naturally be termed alkalosis analogy to the familiar condition called acidosis. In agreent with the more exact definitions of acidosis, we may desigte alkalosis as a pathological condition in which the basic radis in the blood are relatively increased over the acid radicles, regarding carbonic acid. This definition does not specify ether the bases are absolutely increased or the acids not CO_2 decreased or whether both phenomena occur. Nor does it ply that the blood in the body is more alkaline than usual for increased tension of CO_2 may neutralize any tendency toward

[1] Wilson, D. W., Stearns, T., and Janney, J. H., Jr., *Jour. Biol. Chem.*, 5, xxi, 169.

a decreased hydrogen ion concentration and maintain a fin reaction differing little from the normal.

Most of the methods used in the past in the study of acidosis a available for the investigation of a condition such as is describ above. As in acidosis, the problem is complicated by the fact th the variations in the reaction of the blood are never very gre and have always been difficult to determine. As the ordina titration procedures are unsatisfactory, most of the methods study have been indirect.

Several methods have been developed recently which shou be valuable in furnishing the necessary data. Barcroft[2] and h collaborators have shown that the dissociation of oxyhemoglob may serve as a very sensitive index to variations in the reacti of the blood. A new method for the rapid determination of t hydrogen ion concentration of the blood has recently been devis by Levy, Rowntree, and Marriott,[3] which is sufficiently delica to record slight changes in the reaction of the blood.

These two methods, together with the familiar determinati of alveolar carbon dioxide pressure, have furnished evidence support the view that after the removal of the parathyroid glan from dogs there may develop a condition of alkalosis. This alk losis tends to be counteracted by the tetany which soon becom manifest. Acid metabolic products are apparently formed du ing tetany which neutralize the excess of bases and may ev produce an acidosis of varying duration. During the acido periods following the acute attacks, the gross symptoms of teta are usually less evident.

The Dissociation Constant of Oxyhemoglobin.

Barcroft and his collaborators have developed a method f the determination of the dissociation constant of oxyhemoglob and have shown that it is greatly affected by variations in t reaction of the blood. The percentage saturation of hemoglob with oxygen at different oxygen pressures may be determin and a curve constructed to show the relationship of the percenta

[2] Barcroft, J., The Respiratory Function of the Blood, Cambridge, 191

[3] Levy, R. L., Rowntree, L. G., and Marriott, W. M., *Arch. Int. Me* 1915, xvi, 389.

saturation to the oxygen pressure. This curve may be represented by the equation

$$\frac{y}{100} = \frac{Kx^n}{1+Kx^n}$$

where y is the percentage saturation of hemoglobin with oxygen, x is the oxygen pressure, n is the aggregation constant, and K is the dissociation constant of oxyhemoglobin. The aggregation constant, n, for blood is about 2.5 and is not materially changed by ordinary variations in concentration of acids, bases, or salts. This value has been used throughout this investigation. Thus the dissociation constant, K, may be calculated after determining the percentage saturation of hemoglobin in blood in equilibrium with a known pressure of oxygen. When determined at a constant temperature and under comparable conditions the changes in the values of K may indicate variations in the hydrogen ion concentration of the blood examined. Acids decrease the value of K and alkalies increase it. Or, expressed differently, for a definite tension of oxygen, acids decrease the percentage saturation and alkalies increase it.

Two methods of study are readily available. Variations in the actual hydrogen ion concentration of the blood as it exists in the body may be ascertained by determining the values of the dissociation constant of oxyhemoglobin in bloods containing the amount of carbon dioxide which they held when in the body, or, practically, in bloods brought into equilibrium with a pressure of carbon dioxide equal to the alveolar carbon dioxide tension. In order to distinguish these variations from those designated by the terms "acidosis" and "alkalosis," Barcroft has proposed a new nomenclature to describe the results obtained by this method. If the value of K is normal the blood is said to be mesectic; if above normal, pleonectic; if below normal, meionectic. In other words, blood is mesectic, pleonectic, or meionectic according to whether its reaction as it circulates in the body is normal, more alkaline, or more acid than normal.

The second procedure consists in bringing the blood into equilibrium with a constant tension of carbon dioxide and a suitable pressure of oxygen, and determining K. This method eliminates the neutralizing action of the carbonic acid which varies with the

alveolar carbon dioxide tension. The resulting values sho show variations which are comparable to the values obtai by titration methods and indicate changes in the "non-volati acid-base equilibrium or the "reserve alkalinity." Variati from the normal indicate acidosis or alkalosis conditions.

In most of the experiments reported below, the values of t dissociation constant of oxyhemoglobin were determined by t procedure in order to determine whether acidosis or alkal conditions existed. A few determinations were made using t first method.

The determinations[4] were carried out with the large differen blood gas apparatus described by Barcroft and Roberts,[5] follo ing the directions given by Barcroft[6] except for the follow modifications. 3.5 cc. of blood were drawn into a syringe c taining 0.5 cc. of 3 per cent sodium fluoride solution.[7] T mixture was shaken in air for ten to fifteen minutes to rem the CO_2. Blood containing sodium fluoride was found to yi more constant results than defibrinated blood as used by B croft. The determinations were carried out at 37°. Dil ammonia, as used in the method, was introduced into the ri hand bottle of the differential apparatus instead of saturated blo The gas from the tonometer was analyzed after it had been us The oxygen pressure in the tonometer was calculated, correct for temperature and water vapor pressure. No correction the solubility of gases was applied to the calculated percent saturation values as the correction was found to be within errors of the determination. The determinations were usua carried out singly, though many duplicates confirmed the accur of our procedure.

The irregularity of the development of tetany and the rapid with which variations in the dissociation constant of oxyhem globin occurred as the attacks progressed, together with the

[4] The determinations of the dissociation constant of oxyhemoglo were made by Miss Thurlow in Prof. W. H. Howell's laboratory. We w to thank Prof. Howell for the use of his apparatus and his many suggestio

[5] Barcroft, J., and Roberts, F., *Jour. Physiol.*, 1909–10, xxxix, 429

[6] Barcroft, The Respiratory Function of the Blood, Cambridge, 1 Appendix I, p. 292.

[7] The P_H of this solution was 7.4.

avoidable delays necessary for carrying out the determinations, make the data less complete than is desired but it is believed that an idea of the larger variations which may occur can be gained from a composite picture of the experiments reported below. The data from which the following figures were prepared may be found in Tables I to VIII at the end of this paper. The values of K are plotted along the ordinates and time is plotted in days along the abscissae. When several observations were made in one day, the time relations are maintained but the night periods, when the animals were not watched, are omitted. The omission is indicated by a break in the base line and a broken line on the curve. The curves of the alveolar carbon dioxide pressures, also plotted, are discussed later in this paper. The animals were not fed during the experiments.

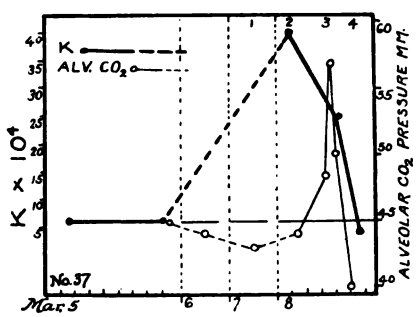

FIG. 1. Experiment 37. Operation Mar. 6. (See Table I.) 1. No tremors; p.m., shivering? 2. Quiet. Very faint tremors. 3. Acute attack of tetany. 4. After attack. Depressed.

Experiment 37.—The dissociation constant of oxyhemoglobin as determined with the blood from Dog 37 showed a normal fasting value of 0.0006. Two days after the removal of most of the thyroid tissue the animal was found in mild tetany. The value of K at this time was 0.0040. The tetany rapidly became acute and at the height of the attack the value of K had dropped to 0.0024. One and one-half hours later, after the attack had subsided, the value of K was slightly below normal.

Analyzing these data in terms of the acid-base equilibrium which they may represent, we find that just preceding an acute attack of tetany, this animal showed a considerable alkalosis. During the acute tetany period the alkalosis diminished. It was completely neutralized

after the acute attack and replaced by a slight acidosis. This probab resulted from the acid metabolic products formed by the extreme m cular activity during the acute tetany period.

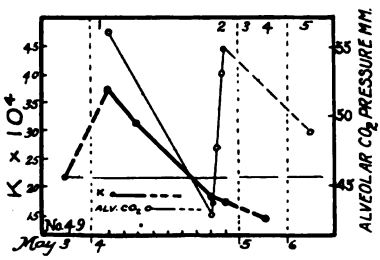

FIG. 2. Experiment 49. Operation May 3. (See Table II.) 1. F tremors. 2. Mild acute tetany. 3. Violent tetany. 4. Mild tremo 5. General tremors.

Experiment 49.—The blood from this animal showed variations simil to those observed with No. 37. The normal value of K was found to unusually high (0.0022) but it had increased to 0.0037 on the day after t operation. The mild tremors did not develop into severe tetany althou the values of K decreased gradually to below normal. A mild attack acute tetany, apparently brought on by excitement, was observed in t afternoon when the value of K was low. After an acute attack on t following day the value of K was still lower. In this experiment, the alk losis condition which developed on the day after the operation was neutr ized by mild continuous tremors.

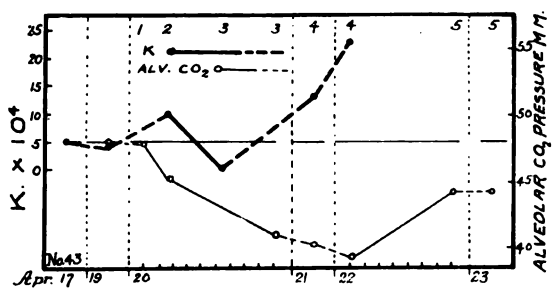

FIG. 3. Experiment 43. Operation Apr. 19. (See Table III.) 1. Fe faint tremors. 2. Acute tetany just started. 3. Prostration. N tremors. 4. Apathetic. No tremors. 5. Marked tremors.

Experiment 43.—A slight alkalosis condition was observed on the da after the operation just before a period of very acute tetany. On accou of the rapidity with which the blood may change in these animals, t

shown by other experiments, the observed value (0.0010) may not represent the maximum alkalosis preceding the attack. During the prostration following the severe tetany, the value of K was found to be very low (0.00008). The values of K indicated the development of a gradually increasing alkalosis during the next two days when no tremors were observed. Tetany became apparent on the second day.

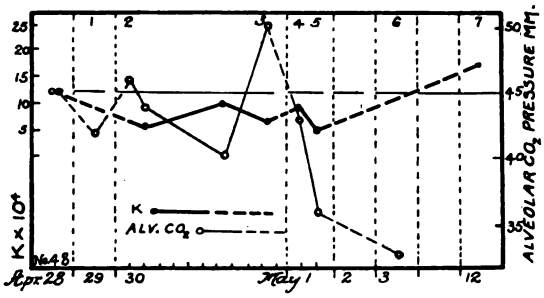

FIG. 4. Experiment 48. Operation Apr. 28. (See Table IV.) 1. Fine tremors. 2. Severe tetany. Improving. 3. Acute tetany, apparently brought on by excitement. 4. Acute tetany. Injected acid. 5. Faint tremors. 6. No tremors. 7. Mild tetany.

Experiment 48.—An acidosis condition was observed following an acute attack of tetany on the second day after the operation. This was neutralized during the day. The value of K again indicated an acidosis during another acute attack. On the next day, a value slightly below normal was observed during acute tetany. An injection of acid caused a relief

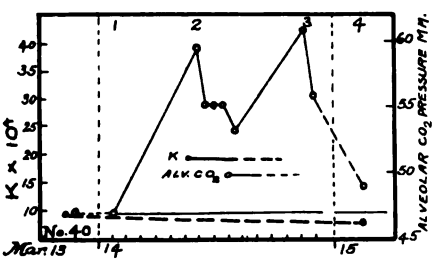

FIG. 5. Experiment 40. Operation Mar. 13. (See Table V.) 1. No tremors. 2. Mild tremors. 3. Tremors increased. 4. Prostration. Mild tremors.

from tetany and a considerable fall in the value of K. Thereafter the animal was treated with acid by mouth and showed only mild tremors for several days. A value of K above normal was observed on April 12 when the dog exhibited mild tetany. Most of the determinations were unfor-

tunately carried out at times which the gross symptoms indicated were not favorable for alkalosis conditions. This fact may account for the lack of high values of *K* during the first few days of the experiment. The relative increase of the acid radicles in the blood after acid injection is clearly demonstrated.

Experiment 40.—A value of *K* slightly below normal was observed on the second day after the operation when the animal appeared to be recovering from an acute attack of tetany.

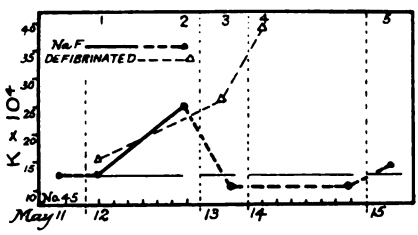

FIG. 6. Experiment 45. Dog with Eck fistula. Operation May 11. (See Table VI.) 1. Shivering, restless. 2. Few contractions. 3. Shivering. 4. Occasional mild tremors. 5. Mild acute tetany.

Experiment 46.—A high value of *K* on the day after the operation indicated a considerable alkalosis when shivering and indefinite contractions suggested a parathyroid insufficiency. During the remainder of the experiment no large variations from the normal were observed in the values of *K* as obtained by our regular method. Defibrinated blood had been found to be less satisfactory than blood to which sodium fluoride had been added

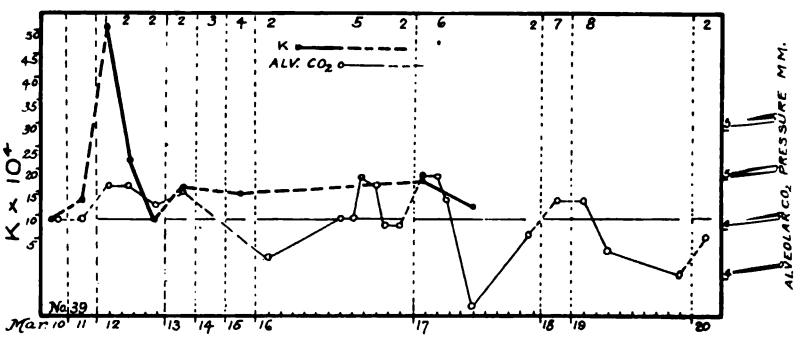

FIG. 7. Experiment 39. Operation Mar. 10. (See Table VII.) 1. Shivering. 2. Mild tremors. 3. No tremors. 4. No tremors. Operation. 5. Acute tetany. 6. Acute tetany. Injected $CaCl_2$ solution. 7. Moderate tremors. 8. Mild tremors. Injected $CaCl_2$ solution.

s an anticoagulant but in this experiment we determined to compare ıe results of the two methods. The normal values of *K* obtained with oth methods were closely comparable but on the following days the debrinated blood furnished rapidly increasing values while the regular etermination gave values slightly below normal. We are unable at presıt to account for the large discrepancy.

Experiment 39.—An extreme alkalosis was observed early on the second ay after the operation. At this time definite tremors were not observed lthough a slight shivering which might be attributed to fright or excitement under ordinary circumstances was apparent. Mild tremors developed uring the day while the alkalosis became completely neutralized. A spontaneous recovery seemed to occur as the tremors disappeared on the following day and the value of *K* was but slightly above normal on March 13 nd 15. The remaining thyroid tissue was therefore removed on the 5th. Acute tetany developed on the following day. A slight alkalosis ·as evident on the morning of the 17th. Another period of acute tetany eveloped and was relieved by injections of $CaCl_2$ solution. An hour fter the last injection the value of *K* had returned nearly to normal.

To gain an idea of the variations in the hydrogen ion concenration of the blood as it existed in the body, two determinations ·ere carried out with blood containing CO_2. The value of *K* btained on the 11th (when the regular determination showed the lood to be about normal) was 0.00026 with blood in equilibrium ·ith a pressure of CO_2 slightly higher than the alveolar CO_2 ·nsion. On the 17th during extreme tetany, the value of 0.00024 ·as obtained from blood in equilibrium with 38 mm. CO_2. The lveolar CO_2 tension at the time was 50 mm. Had this pressure een used in the determination the value would have been conderably lower. These determinations are sufficient to show that, ı the period of acute tetany, the blood was meionectic, *i.e.*, ıore acid than normal. This may account for the panting bserved at this time.

Experiment 42.—This animal did not develop acute tetany for several ays after the operation although mild tremors were apparent on the ·cond day. The rise and fall in the values of *K* during the first four days f the experiment without the development of acute tetany emphasize imilar observations in previous experiments. Notwithstanding the more ›ronounced tremors on the 27th, a considerable alkalosis persisted during he day, increasing to a maximum in the evening. The value of *K* dropped ıuickly after the injection of $CaCl_2$ solution but did not return to normal. .t was slightly lower on the following day but was high again on the next.

Another comparison was made of the variations in the reactic of the blood brought into equilibrium with a gas mixture co taining CO_2 at a pressure approximating that of the alveolar a On the 26th, when the value of K by the regular method was st somewhat above normal, the value of K with blood containir the requisite amount of CO_2 was 0.00019. On the following da although the alveolar carbon dioxide pressure was considerab higher, a value of 0.00031 was obtained. Thus, in spite of t increased content of carbonic acid, the blood was pleonectic more alkaline than on the previous day. This decreased hyd gen ion concentration of the blood in the body suggests an creased irritability of the respiratory center.

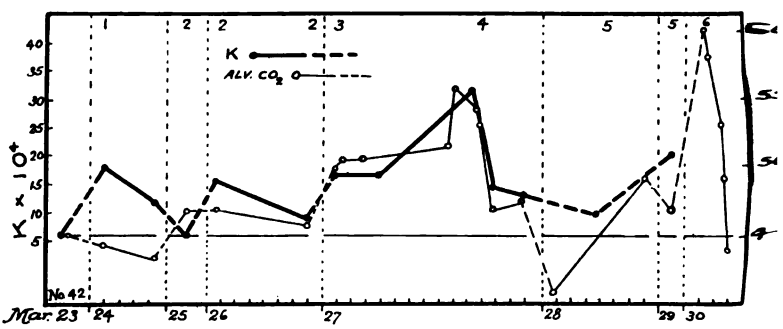

FIG. 8. Experiment 42. Operation Mar. 23. (See Table VIII.) 1. Qui No tremors. 2. Fine tremors. 3. Moderate tremors. 4. Inject $CaCl_2$ solution. 5. Mild tremors. 6. Acute tetany.

The dog appeared at this time to be more quiet than usual an toward evening appeared quite sick and apathetic. This is o interest in connection with Barcroft's[8] observation that "whe the subject feels in normal health, the blood is mesectic, though i may be abnormal. If blood is pleonectic or meionectic, the sub ject feels out of his normal condition and shows symptoms of th change."

Should this prove true, our findings might explain some of th symptoms usually observed in dogs after parathyroidectomy Before attacks the animals appear either uneasy or unusuall

[8] Barcroft, J., Camis, M., Mathison, G. C., Roberts, F., and Ryffel J. H., *Jour. Physiol.*, 1912–13, xlv, p. xlvii.

quiet, which might possibly be explained in accordance with the above results as due to a condition of pleonexy. The depressed condition after the acute attacks may be partly due to meionexy caused by the piling up of the acid metabolic products of the muscle activity.

The following controls were carried out to show the effects of fasting, the operation, and the removal of thyroid tissue. The data are recorded in Table IX.

Experiment 46.—Dog fasted twelve days. The variations in the values of K are within the experimental errors.

Experiment 56.—Lower lobes of both thyroids removed. No appreciable variation in the values of K was noted.

Experiment 51.—One and one-half thyroids removed leaving one visible parathyroid. The values of K increased from 0.0009 to 0.0014–0.0016. This dog developed distemper and soon died so that it is uncertain how much these values may be credited.

Experiment 53.—Two large and two small parathyroids removed (all that were visible). No tetany developed. No increase in the values of K was observed.

From the above experiments we may conclude that fasting and the effects of the operation are not sufficient to cause the experimental variations which have been observed. The removal of thyroid tissue apparently plays no rôle in causing our experimental findings. This should, however, be further verified.

The Alveolar Carbon Dioxide Pressure.

The wide use in the past has shown the value of the alveolar carbon dioxide determinations for giving information concerning the acid-base equilibrium in the blood. It was soon observed after Haldane and Priestley[9] introduced their simple method for the collection and analysis of the alveolar air that in conditions of acidosis the alveolar carbon dioxide pressure was diminished. The total carbonic acid content of the blood is the chief factor governing the maintenance of the normal reaction of that fluid. As the pressure of the carbon dioxide in the alveolar air is dependent on the tension of carbon dioxide in the blood which in turn varies with the total carbonic acid therein, the variations of the

[9] Haldane, J. S., and Priestley, J. G., *Jour. Physiol.*, 1905, xxxii, 225.

latter after the introduction of acids and alkalies may easily b followed by studying the alveolar carbon dioxide pressure.

The use of this method for indicating variations in the acid base relationship in the body may be criticized on several ground: It must be assumed that the excitability of the respiratory cente does not vary during the period of observation. As the reactio of the blood is probably the normal stimulus for the respirator center, a constant irritability must maintain a constant hydroge ion concentration of the blood. In such a case the alveolar CC pressure would be a satisfactory index of the "non-volatile" acid base equilibrium in the blood for the carbonic acid would be re placed by other acids diffusing into the blood and a lowered CC tension result. Any variation in the irritability of the respira tory center would, however, cause a new hydrogen ion concentra tion of the blood to be maintained and therefore a variation i the alveolar CO_2 pressure. Thus, decreased irritability of th respiratory center permits an increased alveolar CO_2 tension an an increased hydrogen ion concentration of the blood after mo phine injections (Straub,[10] Hasselbalch[11]). Hasselbalch an Lundsgaard[12] have shown that the irritability of the respirator center varies inversely with the oxygen tension. Changes in th irritability of the center resulting from pathological condition have been little investigated. Hasselbalch[13] found increase irritability during pregnancy causing low alveolar CO_2 tensions

As the respiration is the means of maintaining the norm reaction of the blood, muscular activity must quickly affect i After short periods of strenuous work the alveolar CO_2 tensic is high (Haldane and Priestley,[14] Douglas and Haldane,[15] Coc and Pembrey[16]) but the excess of CO_2 is removed by increase ventilation. The acid metabolic products formed produce temporary acidosis which apparently persists until they are eith

[10] Straub, W., *Biochem. Ztschr.*, 1912, xli, 419.

[11] Hasselbalch, K. A., *ibid.*, 1912, xlvi, 403.

[12] Hasselbalch, K. A., and Lundsgaard, C., *Skandin. Arch. f. Physio* 1912, xxvii, 13.

[13] Hasselbalch, *ibid.*, 1912, xxvii, 1.

[14] Haldane and Priestley, *loc. cit.*

[15] Douglas, C. G., and Haldane, J. S., *Jour. Physiol.*, 1909, xxxviii, 42

[16] Cook, F., and Pembrey, M. S., *ibid.*, 1912–13, xlv, p. i.

burned up or eliminated by the kidneys. Variations in muscular activity which disturb the equilibrium maintained by a respiratory center of constant irritability must therefore interfere with the method as an indicator of the "non-volatile" acid-base equilibrium of the blood.

Higgins[17] has recently shown that different activities cause slight variations in the alveolar CO_2 tension which he believes cannot be explained as due to variations in the reaction of the blood but to some other agent affecting the respiratory center.

In spite of the numerous objections to the method, it has proven of great value in the past in the study of acidosis. Determinations of the alveolar CO_2 tensions of dogs in tetany have therefore been made with the hope that data might be obtained which could be applied to our present study.

The Plesch[18] method was used to obtain samples of the alveolar air. The analyses were carried out with a small Haldane gas analysis apparatus.[19] The Plesch method consists in rebreathing into a closed rubber bag until the mixture of gases is in equilibrium with the venous blood. A volume of air (about 250–350 cc.) large enough to be nearly completely inspired was placed in a rubber bag, the mask[20] attached and placed over the dog's nose. The clamp was removed and the animal allowed to rebreath the air in the bag for periods varying from twenty to thirty seconds. With normal breathing, variations of from twenty to forty seconds made very slight differences in the results. In our earlier experiments the dogs were placed on their backs on an operating table during the collections but later we found it more convenient to allow them to sit or stand while collecting the sample. Most dogs become accustomed to the procedure very quickly and do not become restless during the collections. Struggling tends to produce high results so that care was always taken to keep the animals quiet. A sufficient number of parallel determinations was carried out to establish their uniformity, two or three usually being sufficient. In this way, duplicates varying 1–2 mm. or

[17] Higgins, H. L., *Am. Jour. Physiol.*, 1914, xxxiv 114.

[18] Plesch, J., *Ztschr. f. exper. Path. u. Therap.*, 1909, vi, 380.

[19] Mr. J. H. Janney, Jr., assisted in some of these determinations.

[20] With the mask devised by Dr. Marriott the determination becomes very satisfactory for use with animals.

less were averaged. The percentage results obtained from th gas analyses were reduced to mm. mercury pressure, correctin for the variation in pressure due to differences in the water vap tensions at the different temperatures of the animal.

Data showing characteristic variations in the alveolar C pressure are given in Tables I–X. Most of these values ha been plotted with the curves of K in the preceding figures.

The most noticeable variations occurred during the extrem attacks of tetany when the alveolar CO_2 tensions usually ro rapidly to a maximum and then fell rapidly. The data of Exper ment 37 show variations which are typical of many other exper ments not reported here. As a period of acute tetany set in, th alveolar CO_2 tension rose from 44 to 48 mm. Ten minutes lat it had reached 57 mm. At this time the tetany was extreme an panting had just begun. Ten minutes after panting started, th pressure had dropped to 50 mm. The alveolar CO_2 pressure w considerably below normal after the attack.

The characteristic rise and fall in the alveolar CO_2 pressu during periods of acute tetany may be observed in most of th experiments reported here. The pressure rose gradually as th tremors became more severe even though little effect on the respir tion was observed. We did not determine whether the ventil tion was increased. Soon after the acute attack began, pantin commenced and the alveolar CO_2 pressure dropped more or le rapidly to a value below normal. Later there was often a cond tion of depression and the tremors had nearly or completel disappeared.

The complication introduced by the muscle tremors renders th high values of little importance as indicating a possible alkalosi They probably represent a piling up of CO_2 faster than can b removed. It is interesting in this connection, however, to no the high pressures which may develop before the gross indicatio of hyperpnea, *i.e.*, panting, develop. The alveolar CO_2 tensio may rise 10 mm. before panting starts.

Even more suggestive are the numerous observations whe the alveolar CO_2 tensions rose to values considerably above no mal and remained for hours without any indications of increase stimulation of the respiratory center or excessive muscular activ ity. Persistently high values were observed in Experiment 3

(Table X) where the alveolar CO_2 pressures were 49–51 mm. during several hours on the day after the operation before the tremors were hardly apparent. After the acute attack the high alveolar CO_2 pressures persisted for an unusual length of time. A tension of 56 mm. was found in Experiment 49 when the animal exhibited but few tremors. Dog 40 gave a series of high tensions for eight hours during which only mild tremors were seen. Other experiments give evidence of high alveolar CO_2 pressures persisting during periods when tremors were mild.

It would seem that these values could hardly be attributed to the muscular activity. They might be due to a depression of the respiratory center which would naturally result in an increased CO_2 tension. But in one of the most striking experiments, No. 42, the value of the dissociation constant of oxyhemoglobin indicates that the respiratory center was, indeed, unusually irritable and the hydrogen ion concentration of the blood in the body definitely decreased, resulting in a condition of pleonexy. These increased alveolar CO_2 tensions may therefore indicate that a condition of alkalosis was present at these times.

The low tensions observed after the attacks may be ascribed to the acidosis conditions resulting from the extreme muscle work. Similar low values have often been observed after muscular activity and ascribed to a temporary acidosis caused by the presence of lactic acid formed on account of the lack of an adequate oxygen supply to the muscles during activity.

A comparison of the variations in the values of K and the alveolar CO_2 pressures makes it apparent that in many respects they fail to show parallelism. It may be seen from many of the curves that the sudden rise and fall of the observed alveolar CO_2 tensions cannot be taken as indicating similar variations in the acid-base equilibrium in the body. This again emphasizes the view point that they are due to extreme variations in the CO_2 production caused by the excessive muscular activity. The slowness with which the respiratory center responds to the rapidly increasing CO_2 tension in several experiments may be due in part to the relative excess of bases in the blood and therefore a slower rate of increase of the hydrogen ion concentration of the blood. The increase in temperature of the animal might be expected to influence the whole discussion by shifting the reaction of the blood

and by affecting the heat regulating mechanism, but its actio is complicated and cannot be closely defined at present.

We have, however, several instances where the alveolar CC tension rose with a relative increase in bases as measured by K In Experiment 39 the alveolar CO_2 tension was above norma when the value of K was high. Experiment 42 showed con tinuously high values on the 27th for both. Parallelism is als evident in Experiment 49.

Such parallelism, as well as the similarity in variation note after the tetany periods and injections, suggests that, except i the extreme tetany periods, the alveolar CO_2 pressures may indi cate alkalosis or acidosis conditions in agreement with the valu of the dissociation constant of oxyhemoglobin. Occasional diver gencies may be noted however.

The injection of acids into the blood stream might naturall be expected to cause a variation in the "non-volatile" acid-bas equilibrium. Evidence supporting this was obtained in Experi ment 48. After the intravenous injection of hydrochloric aci solution the value of K decreased and the alveolar CO_2 pressur fell quickly to 36 mm. The administration of acid by mouth was not sufficient to maintain a subnormal value of K. In anothe experiment the alveolar CO_2 pressure suffered similar variations falling from 43 to 34 mm. after the injection of acid.

A fall in the alveolar CO_2 pressure after calcium administration was noted whenever this procedure was used. In Experiment 3 the pressure dropped quickly to a value much below normal. Th immediate decrease in Experiment 42 was as sudden though no so great, but on the following day a low value was observed. In another experiment the injection of 10 cc. of calcium chlorid solution caused a fall in the alveolar CO_2 pressure from 52 to 3 mm. within an hour. The low value persisted during the day

The decrease in the alveolar CO_2 pressure was accompanied by a fall in the values of K. In Experiment 42 both ran paralle but in Experiment 39 the value of K remained slightly above normal while the alveolar CO_2 tension indicated a considerable acidosis. In Experiment 39 the effects of the calcium injection and the acute tetany can hardly be distinguished with certainty but in Experiment 42 the tremors were mild and, from the study

during the day, there was no reason to expect a sudden fall due to the mild tetany.

Several causes for these variations may be suggested. Calcium salts stimulate the respiratory center[21] and cause an improvement in the circulation by increasing the force and magnitude of the heart beat. Either effect would tend to increase the efficiency of aeration of the blood and produce a lowered CO_2 tension. But the cause of the rapid decrease in the value of K after the introduction of a neutral salt in a determination not influenced by the CO_2 tension is less apparent. We have observed similar variations after the injection of acid. Can it be that the calcium salts injected caused an increase in the acid radicles of the blood?[22] Such an action would account for the variations in the values of K and assist in producing the low alveolar CO_2 tensions. The close parallelism between the action of calcium salts and acids when injected into animals in tetany suggests the possibility that at least part of the beneficial action of the calcium salts may be due to a relative increase in acid radicles caused by their administration.

The Hydrogen Ion Concentration of the Blood.

We have endeavored to supplement our previous findings by means of a method for the determination of the hydrogen ion concentration of the blood devised by Levy, Rowntree, and Marriott.[23] The method consists in dialyzing blood in celloidin sacks against neutral salt solution and determining the reaction of the dialysate by color comparisons with an indicator. The procedure is simple and trustworthy if care is taken to work with controlled solutions in a room free from fumes of acids or alkalies. Bloods were examined as they came from the veins and again after shaking out the excess of CO_2. Variations in the "non-volatile" acid-base equilibrium should become apparent with the second procedure.

[21] Hooker, D. R., *Am. Jour. Physiol.*, 1915, xxxviii, 200.

[22] This might be brought about by the formation of $Ca_3(PO_4)_2$ from the disodium phosphate or possibly $CaCO_3$ from the carbonates thus liberating HCl.

[23] Levy, Rowntree, and Marriott, *loc. cit.*

The method is as follows: 10 cc. of blood were drawn from the jugular vein into a syringe containing 1 cc. of neutral 3 per cent sodium fluoride solution and a glass bead, care being taken to avoid the entrance of air. After shaking to insure complete mixing, 2 cc. were introduced into a freshly rinsed celloidin bag about 5 mm. in diameter and 100 mm. long, and placed immediately in a small test-tube containing 3 cc. of neutral 0.8 per cent NaCl solution. Dialysis was allowed to proceed for five minutes at room temperature, when the bag was removed, five drops of the indicator solution (phenolsulphonephthalein) were added, the test-tube was quickly stoppered and rotated gently to cause complete diffusion of the indicator. The color was compared immediately with standard phosphate solutions of known P_H containing similar amounts of indicator. A duplicate determination was made at the same time. The remainder of the blood was rotated vigorously in a Jena glass beaker for ten minutes to remove the excess of CO_2 and the P_H determined as described above.

Phosphate mixtures showing variations of 0.05 P_H were used as standards. At the ordinary range of blood alkalinity, these differences can easily be detected by comparison after the addition of the indicator. The method as carried out yielded results seldom varying more than 0.05. Usually the duplicates appeared identical or showed differences which were less than 0.05. Though the method must be considered quite rough when compared with the gas chain and the dissociation of oxyhemoglobin methods, we believe that consistent variations of 0.10 in P_H may be considered as real and not due to errors in manipulation or inherent in the method.

The absolute values obtained by the use of blood containing CO_2 may be criticized on the ground that CO_2 had escaped during the determination and the solution had consequently become more alkaline. This objection is valid but is less important than might at first thought be expected, for the diffusion of CO_2 from the liquids seems to be quite slow. A column of blood 3 cm. high was used with a surface only 5 mm. in diameter exposed to the air and protected from air currents by the sides of the test-tube and dialyzing bag. The volume of liquid made more alkaline by the diffusion of CO_2 from the surface was therefore relatively very small and, on account of the buffer action of the mixture,

could influence the reaction of the whole very slightly. We are interested here, however, more in the relative values from day to day than in the absolute figures obtained.

The data obtained by the use of this method may be found in Table XI. The normal variations of P_H, using blood as drawn from the vein, were from 7.3 to 7.4. After bringing the blood into equilibrium with the air, the P_H ranged from 7.6 to 7.75 in different normal animals.

Any variations in P_H in blood as it came from the parathyroidectomized animals were evidently too slight to be recorded with certainty by this method. After shaking the blood, however, small but definite changes were noted. In all cases examined in which tetany developed, an increased alkalinity was observed after removing the excess of CO_2.

In Experiment 54, P_H increased from 7.60 to 7.75 on the first day of tetany and decreased when the tetany became chronic. The increase in Experiment 55 from 7.75 to 7.80+ as tetany developed is less definite. Blood from Dog 49 was more alkaline on the day after the operation and was normal on the following day after an acute attack. These variations run roughly parallel to those indicated by the values of K.

The largest variations in the reaction of the blood were observed in Experiment 45. On the day after the operation, the P_H increased from 7.65 to 8.00 and remained high for several days. The value had returned to normal after a period of chronic tetany. These values, indicating an alkalosis, are more nearly parallel to the values of K as determined with defibrinated blood.

A considerable acidosis was observed in Experiment 48 after the injection of acid, in agreement with the results of the alveolar CO_2 and oxyhemoglobin dissociation methods. After shaking out the CO_2, the P_H was 7.40 instead of the normal value of 7.65. Even the blood dialyzed directly showed an appreciable increase in hydrogen ion concentration.

Experiments 52 and 53 show the constancy of results obtained with animals not developing tetany after an operation.

We may conclude from the results of this investigation that, after parathyroidectomy, there may occur a disturbance in the "non-volatile" acid-base relationship in the body and a condi-

tion of alkalosis result. The alkalosis may be neutralized during the tetany periods which develop. After acute attacks an acidosis often results, probably due to the formation of large amounts of lactic acid during the period of extreme muscular activity. The alkalosis may again develop so that periodic variations in the acid-base equilibrium may accompany the periodic attacks of tetany.

As mild tetany seems to be as efficient in counteracting an alkalosis in some dogs as severe tetany does in others, and as the severity of the tetany bears no apparent relationship to the degree of alkalosis observed, it would appear that other agencies within the body are also active in combating the pathological condition.

Whether the alkalosis condition is in any way the cause for the tetany can hardly be answered definitely from the data at hand. The value of K can apparently tell us nothing as to the type of tetany to expect, for the blood from Dog 39 showed a very high value of K followed by mild tremors and spontaneous recovery; while in Experiment 43 the observed value was but little above normal though the tetany later in the day was extreme. In Experiment 49, an extreme attack of tetany was apparently brought on by excitement though the value of K was slightly below normal. In Experiment 48, no high value was observed though no observations were made before the first acute attack when the largest variation might be expected.

But the data are incomplete and until more frequent observations establish the maximum variations such considerations are of little value. The variability in the development of tetany and the rapidity with which the acute attacks sometimes appear render it difficult to carry out complete experiments. The dogs were often found in tetany in the morning and, at times, handling seemed to stimulate the onset of the attacks.

It is apparent, however, that when the value of K is high tetany tends to lower it and relief from tetany is associated with the fall. An acidosis condition seems to be most favorable for partial or complete relief from tetany. The gross symptoms may completely disappear during the acidosis periods following acute attacks. Artificial acidosis produced by the injection of acid and an apparently similar condition brought about by the injection of calcium salts are likewise important in causing relief from tetany.

SUMMARY.

fter parathyroidectomy, a condition of alkalosis may develop
:h is neutralized by acid products formed by the muscular
vity incident to tetany. An acidosis condition may result
r periods of acute tetany. Periodic variations in the "non-
.tile" acid-base equilibrium seem to accompany the periodic
.cks.

he acidosis condition resulting from acute tetany or the
ction of acid is associated with relief from tetany.

alcium salts, when injected into animals in tetany, lower the
ıe of the dissociation constant of oxyhemoglobin and the
:olar CO_2 pressure, an effect similar to that brought about
the introduction of acids.

'he above conclusions were reached from a study of the varia-
ıs of the values of the dissociation constant of oxyhemoglobin,
alveolar CO_2 pressure, and the hydrogen ion concentration
:he blood from dogs subjected to parathyroidectomy.

TABLE I.

Experiment 37. Female Bull Dog. Weight 11 Kg. Last Fed . Lower Third of One Thyroid Retained.

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	Alveolar CO_2 pressure	
Mar.		*mm.*	*per cent*		*mm.*	
5	9.45 a.m.	29.4	75	6		
	4.00 p.m.	26.9	70	6	45	
6	10.00 a.m.				44	Operation 11.00 a. lar CO_2 pressu 45.
7	10.00 a.m.				43	5.30 p.m. alveo pressure 43 mı ering.
8	9.45 a.m.	12.8	70	40		Very quiet, ve tremors.
	10.30 a.m.				44	Appears sick, v t r e m o r s, ture 38.4°.
	12.30 p.m.				48*	Marked tremors, tion normal.
	12.40 p.m.				57*	Occasional pant starting, tetan ing acute, ten 39°.
	12.50 p.m.				50	Extreme tetany, salivation, ten 40°.
	1.00 p.m.	17.8	76	24		Extreme tetany.
	1.50 p.m.				40	Tetany less ac weak, respirat temperature 4
	2.30 p.m.	37.1	77	4		Weak, depressed dead at 4.00 p.ı

*One determination only.

TABLE II.

Experiment 49. Young Male Cur. Weight 9 Kg. Lower Poles of Each Thyroid Retained.

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	Alveolar CO_2 pressure	
May		*mm.*	*per cent*		*mm.*	
3	10.45 a.m.	17.9	75	22		Operation 11.00 a.m.
4	10.15 a.m.	16.7	81	37	56	Fine tremors.
	12.10 p.m.	17.1	79	31		Fine tremors.
	5.15 p.m.	18.6	73	18	43*	Slight tremors. Mild acute tetany apparently brought on by excitement. Panting and tremors.
					44*	
					48*	
					53*	
	5.55 p.m.				55*	
	6.10 p.m.	19.2	74	17		
5	1.10 p.m.	15.9	59	14		9.00–11.00 a.m. violent tetany. P.m. mild tremors.
6	2.30 p.m.				49	General tremors. 1 gm. HCl *per os* a.m.

*One determination only.

TABLE III.

Experiment 43. Female Bull Dog. Weight 9 Kg. Complete Thyroparathyroidectomy.

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	Alveolar CO_2 pressure.	
Apr.		*mm.*	*per cent*		*mm.*	
17	9.25 a.m.	36.9	80	5		
19	10.10 a.m.	36.3	77	4	48	Operation 10.30.
20	9.00 a.m.				48	Few faint tremors, quiet. Respiration 20, temperature 38.6°, pulse 135. 9.30 tremors increased.
	10.30 a.m.	26.6	79	10	45	Acute tetany just started, panting barely begun.
	2.25 p.m.	69.4	77	0.8		Prostration.
	6.00 p.m.				41	Prostration, weak, no tremors, temperature 36.5°.
21	10.30 a.m.	16.2	59	13	40	Apathetic, weak. 5.30 p.m. same, temperature 37.5°.
22	11.00 a.m.	16.2	71	23	39	Bright, no tremors.
	6.00 p.m.				44	Marked tremors.
23	9.30 a.m.				44	Marked tremors. 0.5 gm. HCl *per os*.
25						Sacrificed.

TABLE IV.

Experiment 48. Female Collie. Weight 15 Kg. Lower Pole of L Retained.

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	Alveolar CO_2 pressure	
Apr.		*mm.*	*per cent*		*mm.*	
28	11.00 a.m.	22.9	75	12	45	Operation 2.30 p
29	10.30 a.m.				41	Fine tremors. tion 33, pulse
	5.00 p.m.				42	Fine tremors, s ing. Temper
30	9.00 a.m.				46	Severe tetany, panting. T 40.7°.
	10.00 a.m.	33.7	80	6	44	Attack subsidin
	3.30 p.m.	18.6	61	10	40	Slight tremors,
	6.20 p.m.	18.6	51	7	50	Acute attack brought citement.
May						
1	9.45 a.m.	18.7	58	9	43	Acute tetany. injected 125 c cent HCl in C NaCl solution
	11.00 a.m.	19.2	44	5	36	Very faint trem
3					33	No tremors May by mouth 1 g 300 cc. water Mild tetany or feeding meat fair condition gestion. A bread and 1 sionally.
12	3.00 p.m.	11.3	43	17		Mild tetany. S ingestion on tetany until 1 dead on 24th.

TABLE V.

Experiment 40. Female Dog. Weight 11 Kg. Lower Third of Left Thyroid Retained.

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	Alveolar CO_2 pressure.	
Mar.		*mm.*	*per cent*		*mm.*	
13	9.35 a.m.	31.8	84	9	47	Operation 10.30.
14	9.00 a.m.				47	No tremors.
	2.45 p.m.				59*	Mild tremors, respiration rapid and slightly labored. No panting.
	3.15 p.m.				55	Mild tetany.
	3.45 p.m.				55	Mild tetany.
	4.15 p.m.				55	Mild tetany.
	5.12 p.m.				53	Mild tetany. Temperature 39.5°, respiration 64.
	10.00 p.m.				61	Mild tetany.
	10.30 p.m.				56	Mild tetany, general tremors, labored respiration 36.
15	9.30 a.m.	29.8	79	8	49	Prostration. General mild tremors. Died about noon.

*One determination only.

TABLE VI.

Experiment 45. Male Dog. Weight 11 Kg. Eck Fistula Established April 3. On Bread and Milk Diet until This Experiment Started. In Good Condition. Lower Pole of Right Thyroid Retained.

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	
May		*mm.*	*per cent*		
11	10.35 a.m.	19.4	69	13	Operation 11.00 a.m.
12	9.40 a.m.	17.4	66	15	Shivering, restless. Defibrinated blood.
	9.50 a.m.	20.1	70	13	
	3.50 p.m.	11.8	55	25	Some contractions but no definite tremors.
13	11.25 a.m.	12.9	61	26	Some contractions but no definite tremors. Defibrinated blood.
	12.10 p.m.	11.3	32	11	Some contractions but no definite tremors.
14	10.05 a.m.	13.1	71	39	Occasional mild tremors. Defibrinated blood.
	4.00 p.m.	20.5	67	11	
15	9.50 a.m.	19.0	69	14	Mild acute tetany.

Lived until the 26th showing occasional periods of mild tremors. Fed after the 17th.

TABLE VII.

Experiment 39. Female Dog. Weight 10 Kg. Posterior Ha
Left Thyroid Retained.

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	Alveolar CO_2 pressure.	
Mar.		*mm.*	*per cent*		*mm.*	
10	1.15 p.m.	23.2	69	9	46	Operation 4.00 p.m
11	5.50 p.m.	22.0	75	13	46	
12	10.00 a.m.	13.7	78	51	49	Shivering.
	11.40 a.m.	23.0	85	22	49	Mild tremors.
	1.40 p.m.	28.0	80	9	47	Mild tremors.
13	12.00 m.	24.8	83	16	48	Mild tremors. 5.3 lar CO_2 48 mm.
14						No tremors.
15	11.30 a.m.	25.2	83	15		4.00 p.m. removed ing thyroid tissu
16	11.00 a.m.				42	Mild fine tremors.
	5–5.30 p.m.				46	Tremors. Occa panting period. 150, temperature
	6.00 p.m.				46	Acute tetany. (ous mild pantin piration 200, t ture 38.5°.
	6.40 p.m.				50	Acute tetany.
	7.40 p.m.				49	Acute tetany. Res 240, temperatur
	8.30 p.m.				45	Tetany less acute alveolar CO_2 sam perature 39.9°.
	10.15 p.m.				45	Apathetic, weak. tremors. Respir pulse 65.
17	9.25 a.m.	28.7	89	18	50	Marked tremors.
	10–11 a.m.				50–51	Acute tetany. In cc. 5 per cent Ca tion 11.00 a.m.
	11.30 a.m.				48	Tetany less but sti ed. 12.35 p.m. 8 cc. $CaCl_2$ soluti
	1.45 p.m.	30.4	86	12	37	Depressed, no Respiration 30, t ture 37°.
	6.25 p.m.				44	Fine tremors.

TABLE VII—*Continued.*

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	Alveolar CO_2 pressure.	
Mar.		*mm.*	*per cent*		*mm.*	
18	11.00 a.m.				48	Moderate tremors. 4.00 p.m. alveolar CO_2 45 mm.
19	9.00 a.m.				48	Mild tremors. Depressed. Injected 5 cc. $CaCl_2$ solution 10.00 a.m.
	10.40 a.m.				43	5.00 p.m. alveolar CO_2 41 mm.
20	9.00 a.m.				44	Mild tremors, depressed, weak.
	4.00 p.m.				46	
11	12.25 p.m.	34.8	65	2.6		CO_2 49 mm. alveolar CO_2 46 mm.
17	10.55 a.m.	27.0	48	2.4		CO_2 38 mm. alveolar CO_2 50 mm.

TABLE VIII.

Experiment 42. Young Female Bull Dog. Weight 9 Kg. Last Fed Posterior Third of Left Thyroid Retained.

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	Alveolar CO_2 pressure.	
Mar.		*mm.*	*per cent*		*mm.*	
22	9.30 a.m.				45	
23	10.15 a.m.	30.9	75	6		Operation 10.30 a p.m. alveolar C
24	10.55 a.m.	23.4	83	18	44	Quiet, no tremors
	2.25 p.m.	25.1	80	12	43	Alveolar CO_2 5.3(8.00 p.m. 48, 1 45 mm.
25	12.00 m.	25.3	67	6	47	Fine general tren veolar CO_2 5.3(mm.
26	9.25 a.m.	22.1	79	16	47	Fine general Bright. Pulse piration 16, ter 38.5°.
	4.00 p.m.	27.6	79	9	46	Fine general tren
27	9.45 a.m.	28.2	88	17	50	Tremors more pr(Respiration 56 pulse 100. Alv 10.00 a.m. 51, 1 51 mm.
	1.50 p.m.	23.0	81	17		5.30 p.m. Respi pulse 128, alv(52. 6.00 p.m. 5
	7.35 p.m.	17.3	80	32	54	Injected 11 cc. 5 $CaCl_2$ solution, 9 cc. Alveolar p.m. 53 mm. pu
	8.45 p.m.	17.3	64	14	47	Respiration 28, regular 60. Tre
	10.57 p.m.	18.1	65	13	48	
28	10.00 a.m.				41	Respiration 11, pu
	12.30 p.m.	18.8	61	10		Mild general trem p.m. alveolar mm.

TABLE VIII—*Continued.*

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	Alveolar CO_2 pressure.	
Mar.		*mm.*	*per cent*		*mm.*	
29	11.45 a.m.	17.3	71	20	47	Tremors. Temperature 38°.
30	8.45 a.m.					Tremors, quiet, panting started at 9.00 a.m.
	9.15 a.m.				60*	Severe tetany, stiff, restless, salivation, temperature 41.5°, respiration 195.
	9.20 a.m.				57*	
	10.27 a.m.				53*	Pulse 144, temperature 43.4°, respiration 80.
	10.35 a.m.				49*	10.40 a.m. alveolar CO_2 44* mm. Died 11.00 a.m.
26	2.00 p.m.	22.9	32	1.9		CO_2 47 mm. alveolar CO_2 46 mm.
27	11.50 a.m.	15.8	24	3.1		CO_2 58 mm. alveolar CO_2 51 mm.

*One determination only.

TABLE IX.

Experiment 56. Male Terrier. Lower Lobes of Thyroids Remo All Visible Parathyroids Retained.

Date.	Time.	O_2 pressure.	Saturation.	$K \times 10^4$	
June		*mm.*	*per cent*		
3	4.00 p.m.	15.7	48	9	
7	9.45 a.m.	17.9	55	9	Operation.
8	2.00 p.m.	16.2	57	12	
9	2.15 p.m.	33.7	86	9	

Experiment 46. Male Cur. Last Fed April 29. Fasted.

May					
5	10.45 a.m.	16.2	54	11	
8	11.00 a.m.	19.6	68	12	
11	2.20 p.m.	24.5	79	13	

Experiment 53. Young Male Terrier. Weight 8.5 Kg. Hyperthy Removed Four Parathyroids (All That Were Visible).

May					
31	9.45 a.m.	17.4	60	12	
	11.35 a.m.	16.1	63	16	Operation 3 p.m.
June					
1	2.00 p.m.	17.5	58	11	No tremors. Lively.
2	9.45 a.m.	15.8	62	16	" " "
	11.50 a.m.	15.9	58	14	" " "
3	2.10 p.m.	18.4	60	10	" " "

Experiment 51. Male Cur. Left Thyroid and Lower Pole of R Thyroid Removed. One Visible Parathyroid Retained.

May					
18	10.15 a.m.	23.9	71	9	Operation 2.30 p.m.
19	9.28 a.m.	18.9	69	14	No tremors.
	3.30 p.m.	19.0	70	15	No tremors.
20	10.45 a.m.	18.7	63	11	No tremors. Nose infect
21	10.55 a.m.	14.5	56	16	Developed distemper. I 25th. Middle lobe of consolidated.

TABLE X.

Experiment 31. Female Bull Dog. Weight 8 Kg. Operation Feb. 15. Lower Pole of Left Thyroid Retained.

Date.	Time.	Alveolar CO_2 pressure.	
Feb.		*mm.*	
16	10.45 a.m.	47	Very faint tremors.
	12.00 m.	49	
	4.20 p.m.	51	Faint general tremors. Sleepy. Respiration deep and slow. Temperature 40°.
	4.45 p.m.	53*	Tremors more marked. Respiration more rapid, 33. Restless.
	5.00 p.m.	56*	Tremors increasing. Occasional panting. Restless.
	5.20 p.m.	56*	Panting more frequently.
	5.35 p.m.	53	Panting continuous. Respiration 260. Restless, stiff. Extreme tetany. Temperature 40.6°.
	6.00 p.m.	53	Tetany less acute. Respiration 80. Temperature 40.1°.
	8.30 p.m.	53	Faint tremors. Muscles in tone. Respiration slow and labored. Temperature 39.2°.
17	9.30 a.m.	43	Mild tremors. 1.30 p.m. acute tetany, injected 38 cc. 0.5 per cent HCl in water.
	5.30 p.m.	34	No tremors. 11.30 p.m. no tremors.
18	9.45 a.m.	46	Fine tremors. Temperature 39.8°.
	11.15 a.m.	48	Acute tetany. Temperature 40.3°.
	3.00 p.m.	38	After the attack. Depressed.
	5.00 p.m.	44	Mild tremors. 6.00 p.m. alveolar CO_2 44 mm.

* One determination only.

TABLE XI.

The P_H of the Blood from Normal and Parathyroidectomized Ani

Experiment 54. Male Terrier. Weight 10 Kg. Four Parathyroi Removed.

Date.	P_H direct.	P_H after shaking.	
May 31	7.35	7.60	Operation.
June 1	7.35	7.70	
2	7.40	7.70	2.00 p.m. Faint tremors.
3	7.40	7.70	9.30 a.m. Faint tremors.
3	7.35+	7.75	5.00 p.m. Considerable tr
4	7.35	7.70	Few mild tremors.
5		7.65	Chronic tetany.

Experiment 55. Male Cur. Weight 5.2 Kg.

June 1	7.40	7.75	Operation.
2	7.35+	7.80+	Mild occasional tremors.
3	7.35	7.70	No tremors. Depressed. during night.
4	7.35	7.70+	No tremors. Depressed.

Experiment 49. Male Cur. Lower Poles of Each Thyroid Retain

May 3	7.40	7.70	Operation.
4	7.40	7.80	9.30 a.m.
5	7.35	7.70	3.00 p.m. Acute tetany 11.00 a.m.

Experiment 45. Eck Fistula Dog. Lower Pole of Right Thyroid Reta

May 11	7.35	7.65	Operation.
13	7.40+	8.00	Defib.* 8.10. 9.00 a.m. No
14			Defib. 7.80 in a.m.
14			4.00 p.m. Defib. 8.00. Ton
15	7.35	7.90	Ton. 7.90. Tetany marked
25	7.40	7.65−	Chronic tetany.

*Defib. = defibrinated blood used. Ton. = blood from tonomete

TABLE XI—*Continued.*

Experiment 48. Female Collie. Lower Pole of Left Thyroid Retained.

Date.	P_H direct.	P_H after shaking.	
Apr.			
29	7.30	7.65	Operation Apr. 28.
May			
1	7.20	7.40	Ton. 7.40. After acid injection.
3	7.20—	7.40	No tremors. Acid by mouth.

Experiment 52. Male Bull Dog.

May			
21	7.30	7.70	Operation, nothing removed.
28		7.65	

Experiment 53. Four Parathyroids Removed.

May			
31	7.35	7.75	Tpn. 7.80. Operation.
June			
1	7.35	7.75	No tetany developed.
2	7.35	7.70	June 3. Ton. 7.75.

E EXCRETION OF ACIDS AND AMMONIA AFTER PARATHYROIDECTOMY.

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nerous observations have been made on the urine of dogs parathyroidectomy which suggest that a state of acidosis

Acetoacetic and lactic acids have sometimes been found. phenomena, however, fail to support that view and may even re easily explained on the assumption that there is a relative of alkali in the body, an alkalosis. That both conditions occur at different periods in the course of tetany seems ble.

have shown in a previous communication[1] that acids, when istered to dogs in parathyroid tetany, relieve the symptoms. tive excess of alkali (neglecting CO_2) in the blood at certain s after parathyroidectomy is indicated by the values of the ation constant of oxyhemoglobin, alveolar CO_2 pressure, and drogen ion concentration of the blood.[2] These studies make ear that the alkalosis is primary, and that the acidosis de- only secondarily during the attacks of tetany and may give to another alkalosis period unless the tetany is sufficient ntain it. In the present paper data are presented concern- e ammonia and acid excretion and the hydrogen ion concen- n of the urine which may furnish additional details concern- e variations taking place within the body.

lson, D. W., Stearns, T., and Janney, J. H., Jr., *Jour. Biol. Chem.*, xi, 169.

paper just preceding this.

Dogs were placed in metabolism cages and the urine was collected in two[3] periods daily by catheterization, extreme care being taken to guard against cystitis. A sterile catheter and vaginal dilator were used after thoroughly washing the external surfaces and the vagina with warm sterile water or very dilute bichloride solution. No food was given the animals after the operations. This procedure may have diminished the variations in our findings on account of the tendency toward acidosis by fasting and the less favorable condition for tetany development. After a suitable fore-period, the parathyroids were removed with varying amounts of thyroid tissue and the animals kept under observation. Water was usually given by stomach sound twice daily.

The following methods were used: total nitrogen, Kjeldahl; ammonia, Folin; acid excretion, Henderson and Adler.[4] On account of the pigment in the urine which was apparent even after the dilution of 10 cc. of urine to 200 cc., a second flask of diluted urine was placed behind the standard and a flask of distilled water behind that in which the titration was carried out. In this way, color comparisons were much more exact, especially where the urine volume varied considerably. The hydrogen ion concentration of the urine was determined by Henderson's method.[5] Instead of making color comparisons in 250 cc. flasks, test-tubes of uniform bore were used. 2 cc. of urine were introduced, 20 cc. of water and a drop of sodium alizarine sulphonate solution added. After thorough mixing, the color was compared with a standard phosphate solution containing the same amount of indicator. A test-tube containing diluted urine was placed behind the standard as described above. If the urine had a P_H of 7.0 or greater phenolsulphonephthalein was used as the indicator.

Experiments were carried out on six dogs from which the parathyroid glands were removed together with most of the thyroid

[3] Short periods were used on account of the rapid changes known to occur in the animals as tetany develops. The animals were catheterized at 9.00 a.m. and 6.00 p.m. daily.

[4] Henderson, L. J., and Adler, H. M., *Jour. Biol. Chem.*, 1909, vi, xxxviii. See also Adler, H. M., and Blake, G., *Arch. Int. Med.*, 1911, vi, 479.

[5] Henderson, L. J., *Jour. Biol. Chem.*, 1911, ix, 403. Henderson, L. J., and Palmer, W. W., *ibid*, 1912-13, xiii, 393.

tissue. The lower pole of one thyroid was left intact in all experiments except No. 43 which was a complete thyroparathyroidectomy. In three experiments, Nos. 37, 40, and 43, the animals quickly developed acute characteristic tetany. Dog 42 developed tetany which did not become acute for several days. Dog 39 apparently made a spontaneous recovery after showing mild symptoms of parathyroid insufficiency. A second operation was performed at which the remaining thyroid tissue was removed and acute tetany soon developed. Dog 48 exhibited rather unusual symptoms. She was often observed lying in the cage with tremors hardly perceptible but panting and restless, with legs somewhat tense, and apparently suffering from a moderately acute attack of tetany. The lack of severe tremors was noticeable during most of the experiment.

The data obtained from the examination of the urine may be found in Tables I–VI. As the urine was collected twice daily in periods of unequal duration, the data depending on volume are calculated and recorded in terms of the average hourly excretion in order that the figures may be directly comparable.

Urine Volume.—The urine volumes may not be strictly comparable as the water intake may have varied because water was always available for these animals. The portion given by stomach sound was undoubtedly all that was taken during some periods. The urine volumes were usually low at or before the beginning of tetany and increased as the tetany progressed. High volumes were maintained throughout Experiment 48 but maximum values occurred after the acute tetany and acid injection. The specific gravities showed no very characteristic variations.

Total Nitrogen.—The average hourly excretion of total nitrogen fell gradually after feeding was discontinued but rose as tetany developed. The output was considerably increased in the periods after the acute attacks. This increase cannot be attributed to the rise in the ammonia elimination only, for the maximum values are not associated with comparable increases in the percentage ammonia outputs.

Hydrogen Ion Concentration.—The hydrogen ion concentration of the urines decreased after the operations in all of the experiments except No. 48. In this experiment, the urine was more

acid than is usually observed in dogs.[6] The normal value of P_H during the feeding period was 6.0. The urine became more acid after the operation, reaching a maximum (P_H = 5.4) after several days of acid ingestion. The other experiments seem to illustrate the characteristic variations. The average P_H of the urine obtained from the fasting dogs in our experiments was 6.4. Solutions of this P_H are acid to litmus. In Experiment 40, the urine became quite strongly alkaline (P_H = 8.2 +) in the period after the operation. A similar change was observed in Experiment 42 (P_H = 7.8) after the operation. These alkaline urines may hardly be attributed to fermentation or excessive ammonia elimination for their content of ammonia was unusually low. The urine from Dog 43 became less acid on the day after the operation and was alkaline in the afternoon (P_H = 7.9). The excretion of alkaline urine after the acute tetany of the morning is unique. The increased elimination of acids characteristic of the tetany periods seems to have been delayed. The urine became acid during the next day. The urine from Dog 40 was acid on the 15th when she appeared to be recovering from an acute attack of tetany. The urine in Experiment 42 remained very faintly acid during most of the experiment after the first period following the operation. No very characteristic changes in the reaction of the urine were observed in Experiment 39.

The urine tends to become less acid after parathyroidectomy and may even show an alkaline reaction. An increased acidity results after tetany.

Ammonia.—The ammonia excretion fell suddenly after the operation in most of the experiments and remained at a low value until tetany became manifest, when it rose abruptly. The low output was usually associated with a low ratio of NH_3—N to total N and the increased excretion following tetany resulted in an increased ratio. In Experiment 40, the average hourly excretion in the period after the operation was but one-fifth that of the previous period. The ammonia ratio at this time was only 1.2 per cent. Twenty-four hours later the ammonia ratio was 2.08 per cent. On the morning of the 15th, apparently after

[6] Albumin was found in the urine on the morning of May 1 and in apparently increasing amounts during the two periods following. Other urines in this experiment were not examined for albumin.

acute tetany, the ammonia excretion showed a fourfold increase and the ratio to total N rose to a maximum value. Similar variations occurred in Experiment 43 although the ammonia ratios showed no large variations from the normal values. The excretion of ammonia was unusually high after several days of tetany. The low output following the operation in Experiment 42 was accompanied by a low percentage value. Rather high relative and absolute values were observed on the morning of the 25th as tetany became apparent. The maximum excretion occurred during the two periods before death after a number of days of tetany. The ammonia ratios at this time, 11.88 per cent and 10.22 per cent, were among the highest observed in this work. In Experiment 39, the output decreased gradually to a minimum during the period of parathyroid insufficiency and then rose as recovery took place. The second operation was followed by a moderate decrease in elimination for two periods. After the subsequent rise, no characteristic variations occurred. Experiment 48 was unusual in showing an almost unbroken rise in the ammonia output. The maximum elimination occurred during the two periods following the acid injection on the second of which the ammonia ratio was 12.9 per cent. The ammonia excretion fell on the last two days even though 1 gram of hydrochloric acid was administered daily.

Acid Excretion.—The acid excretion ran nearly parallel with the ammonia output. In all of the experiments reported here there was a sudden diminution in the elimination of acids during the period following the operation. The decreased elimination usually persisted for several periods and then rose as tremors and acute tetany developed. The decreased excretion of acids resulting in an alkaline urine in Experiment 43 after the acute tetany during the morning of the 20th seems unusual. If an excess of acids was developed at that time, their excretion was delayed for several hours.

The excretion of acids as determined by titration ran only roughly parallel to the hydrogen ion concentration of the urine. Low titration values were usually associated with a decreased hydrogen ion concentration. They were never very large with alkaline urines although the hydrogen ion concentration occasionally was quite low. Considerable variation occurred in Ex-

periment 42 when the P_H remained nearly constant. The low titration values in Experiment 48, May 2, p.m. and 3, a.m., in urines with high hydrogen ion concentrations suggest that stronger acids than monosodium phosphate were being excreted.

Total Acid Excretion.—As the variations in the excretion of acids and ammonia ran quite parallel in these experiments, the total acid excretion, represented by the sum of the equivalent amounts of these two constituents, showed similar changes. The variations observed after operation ran roughly parallel to the urine volume, both being low before tetany and increasing after tetany developed. Many exceptions are in evidence however.

The total acid excretion hardly accounted for all of the acid administered in Experiment 48 on May 2, p.m. An equivalent of 135 cc. of $\frac{N}{10}$ HCl was given twice daily while only 102 cc. were excreted during this period. An equivalent of 153 cc. was eliminated during the following period. When it is considered that an equivalent of 150–250 cc. of $\frac{N}{10}$ acid was excreted under normal conditions and might still be expected to be eliminated, the deficit becomes more noticeable.

The results of some of the experiments reported above should be comparable to data presented in the previous paper as the same animals were used in each. Individual comparisons are difficult, however, on account of the rapid variations and the incompleteness of the data.

Data have been presented which show, in general, that after parathyroidectomy there is a marked diminution in the excretion of acids and ammonia accompanied by a decrease in the ratio of NH_3-N to total N, and in the hydrogen ion concentration of the urine. After the development of tetany, the excretion of acids and ammonia increases as well as the ammonia ratio and the hydrogen ion concentration of the urine. Greenwald,[7] and Underhill and Saiki[8] have reported experiments in which neutral or alkaline urine was excreted after parathyroidectomy without an increase in ammonia. Cooke[9] found alkaline urine but the high ammonia output and ratio arouse suspicion that bacterial action

[7] Greenwald, I., *Am. Jour. Physiol.*, 1911, xxviii, 103.
[8] Underhill, F. P., and Saiki, T., *Jour. Biol. Chem.*, 1908–09, v, 225.
[9] Cooke, J. V., *Jour. Exper. Med.*, 1911, xiii, 439.

may have occurred. The following facts may be considered in attempting an explanation for these variations.

Studies on the urine in conditions of acidosis have shown that characteristic changes occur. Walter[10] observed many years ago that the ammonia output was increased after the ingestion of acids. From the accumulated data, especially the newer and more exact observations of Henderson[11] and others, the characteristic urine changes in acidosis may now be described as an increase in ammonia and its relation to total nitrogen, increased acidity both as expressed by titration values and the hydrogen ion concentration and increased phosphate elimination unless the phosphate store is depleted. The excretion of ammonia, acids, and phosphates may run roughly parallel for a time.

With the ingestion of alkali, the acidity falls and the urine may become alkaline. A decrease in the ammonia output is characteristic. Janney[12] was able to cause the daily ammonia elimination to diminish to 0.04 gram, which was 0.4 per cent of the total nitrogen, by the ingestion of sodium bicarbonate. These variations in the excretion of ammonia are so characteristic that they have been taken as an index of changes occurring within the body. As a part of the mechanism for the maintenance of neutrality within the body, ammonia combines with a portion of the acids formed and is excreted. In acidosis, when an excess of acids is formed, the ammonia output is increased; when there is an excess of "fixed alkali" in the body, as after excessive ingestion of sodium bicarbonate, the ammonia elimination is diminished.

These relationships might possibly be altered by an unusual abnormality in kidney function. Acidosis conditions due to renal changes have been suggested by Henderson and Palmer.[13] No indications of renal insufficiency have been noted after parathyroidectomy. We have found normal values for the blood urea and the phthalein excretion during tetany in two of our animals.[14]

Analyzed in accordance with the observations mentioned above,

[10] Walter, F., *Arch. f. exper. Path. u. Pharmakol.*, 1877, vii, 148.

[11] Henderson, *loc. cit.*

[12] Janney, N., *Ztschr. f. physiol. Chem.*, 1911–12, lxxvi, 99.

[13] Henderson and Palmer, *Jour. Biol. Chem.*, 1915, xxi, 37.

[14] Blood urea, 33 mg. per 100 cc.; 55 mg. per 100 cc. three hours after feeding meat. Phthalein excretion, 70 per cent in two hours after several days of tetany.

the data presented in this paper indicate that there is a relative excess of "fixed alkali" in the blood, *i.e.*, an alkalosis, soon after parathyroidectomy. This alkalosis is apparently not due to an excess of circulating ammonia, as the ammonia excretion is at a minimum during the periods of decreased acidity of the urine. The ratio of NH_3-N to total N may be unusually low at the same time. Also, there is no satisfactory evidence to show that the ammonia content of the blood is above normal. The variations are strikingly similar to those observed after the ingestion of sodium bicarbonate.

Greenwald[15] first emphasized the fact that the phosphate excretion in dogs is decreased during the first few days after parathyroidectomy. There is apparently an increase in the inorganic phosphates of the blood at the same time.[16] This decreased elimination of phosphates seems to run parallel with our observations of decreased excretion of acids and may well be interrelated in that the low acid output may be due to a decreased excretion of monosodium phosphate which is the chief acid constituent of the urine.

Henderson[17] has shown that an experimental acidosis in rabbits causes an increased elimination of phosphates and suggested that the excretion of acid phosphate may be one of the means for removing acids from the body. This, together with the old observation of Maly,[18] that the monobasic phosphate diffuses much more rapidly than the dibasic phosphate, may be reversed to account for the retention of phosphates during the alkalosis period after parathyroidectomy. There may also be a selective retention to aid in neutralizing bases in the body.

The retention of phosphates may not be accompanied by a retention of sodium and potassium (Greenwald) so that the excretion of these two bases, with the increased elimination of calcium[19] and magnesium[20] may account for the decreased hydrogen ion concentration of the urine and the low titration values. These

[15] Greenwald, *Jour. Biol. Chem.*, 1913, xiv, 363.

[16] Greenwald, *ibid.*, 1913, xiv, 369.

[17] Fritz, R., Alsberg, C. L., and Henderson, L. J., *Am. Jour. Physiol.*, 1907, xviii, 113.

[18] Maly, R., *Ztschr. f. physiol. Chem.*, 1877–78, i, 174.

[19] MacCallum, W. G., and Voegtlin, C., *Jour. Exper. Med.*, 1909, xi, 118.

[20] Cooke, *ibid.*, 1910, xii, 45.

facts also suggest the possibility of a disturbance in the equilibria of the inorganic radicles in the body.

With the development of tetany, the urine picture changes quickly. The increased excretion of acids and ammonia, together with high values of the ammonia ratio, suggests an acidosis. The increased acidity is probably due (in part) to the excretion of the lactic acid formed in the muscles during tetany. Cooke[21] found lactic acid in the urine of dogs during tetany and we observed the same in one experiment.

SUMMARY.

After parathyroidectomy in dogs, there is usually a sudden diminution in the excretion of acids and ammonia and a decrease in the ammonia ratio and the hydrogen ion concentration of the urine. With the development of tetany, the elimination of acids and ammonia increases, accompanied by increased values of the ammonia ratio and the hydrogen ion concentration of the urine.

These variations may indicate that an alkalosis condition results after parathyroidectomy but is neutralized by the tetany which develops. After acute or chronic tetany, an acidosis may occur.

TABLE I.

Experiment 37. Female Bull Dog. Weight 11 Kg. Last Fed Mar. 2. Lower Third of One Thyroid Retained. 150 Cc. Water Given per Os Twice Daily after Operation.

Average Hourly Excretion.

Date.	Volume.	Acid. cc. of 0.1 N solution.	
Mar.	cc.		
4, p.m.	20.0	3.52	
5, a.m.	4.8	1.76	
5, p.m.			
6, a.m.	15.1	2.72	Operation 11.00 a.m.
6, p.m.	33.6	1.68	
7, a.m.	11.7	1.82	
7, p.m.	6.9	0.45	Shivering.
8, a.m.	9.2	1.23	Quiet. Faint tremors.
8, p.m.	4.2	0.29	12.30 p.m. acute tetany. Found dead at 4.00 p.m. Period calculated as 6 hrs.

[21] Cooke, *Jour. Exper. Med.*, 1911, xiii, 439.

TABLE II.

Experiment 40. Female Dog. Weight 11 Kg. Lower Third Left Thyroid Retained. 150 Cc. Water Given per Os Twice Daily.

Average Hourly Excretion.

Date.	Volume.	Total N.	$\frac{NH_3\text{-}N}{\text{Total N}}$	Acid.	NH_3	Total acid.	P_H	
				cc. of 0.1 N solution.				
Mar.	*cc.*	*gm.*	*per cent*					
11, p.m.	18.0	0.605	3.98	2.44	17.22	19.66	6.6	
12, a.m.	15.1	0.375	6.94	4.44	18.60	23.04	6.2	
12, p.m.	26.1	0.183	4.91	1.12	6.40	7.52	6.8	
13, a.m.	11.2	0.120	7.15	2.02	6.16	8.18	6.3	
13, p.m.	3.3	0.140	1.20	−0.81	1.21	0.40	8.2+	Operation 10.30 a.m.
14, a.m.	7.5	0.144	4.80	0.26	4.96	5.22	7.0	No tremors.
14, p.m.	6.9	0.198	2.08	0.04	2.94	2.98	7.2	2.00 p.m. mild tremors. 10.40 p.m. tremors increased.
15, a.m.	9.6	0.258	7.40	3.18	13.55	16.73	6.6	Prostration. (Following acute attack?)
15, p.m.	34.0	0.356	5.27	5.27	13.34	18.61	7.0	Died about noon.

TABLE III.

Experiment 43. Female Bull Dog. Weight 9 Kg. Complete Thyroparathyroidectomy. 150 Cc. Water Given per Os Twice Daily after Operation.

Average Hourly Excretion.

Date.	Volume.	Specific gravity.	Total N.	$\frac{NH_3\text{-}N}{Total\ N}$	Acid.	NH_3	Total acid.	P_H	
					cc. of 0.1 N solution.				
Apr.	cc.		gm.	per cent					
15	17.7		0.368	8.11	5.85	21.33	27.18	6.6	Fed regular diet.
16	18.4	1.032	0.371	6.13	3.78	16.21	19.99	6.8	" " "
17	23.6	1.024	0.322	6.15	2.29	14.17	16.46	7.2	" " "
18	12.3	1.033	0.292	5.17	2.42	10.75	13.17	7.0	Fed bread only.
19, a.m.	14.0	1.028	0.146	9.75	3.37	10.17	13.54	6.4	Fasting started.
19, p.m.	3.8	1.044	0.133	5.30	0.76	5.02	5.78	6.4	Operation 10.00 a.m.
20, a.m.	4.5	1.026	0.102	8.20	0.61	5.97	6.58	6.6	Mild tremors.
20, p.m.	14.1	1.017	0.117	6.05	−0.14	5.08	4.94	7.9	11.00 a.m. acute tetany followed by prostration.
21, a.m.	42.7	1.011	0.258	4.14	2.77	7.68	10.45	7.2	Weak, no tremors.
21, p.m.								6.8	Fine tremors.
22, a.m.									Mild tremors.
22, p.m.	12.3	1.020				23.45		7.2	Urine contained some blood.
25									Sacrificed.

TABLE IV.

Experiment 42. Young Female Bull Dog. Weight 9 Kg. Last Fed Mar. 19. Posterior Third of Left Thyroid Retained. 150 Cc. Water Given per Os Twice Daily.

Average Hourly Excretion.

Date.	Volume.	Specific gravity.	Total N.	$\frac{NH_3\text{-}N}{Total\ N}$	Acid.	NH_3	Total acid.	P_H	
					cc. of 0.1 N solution.				
Mar.	cc.		gm.	per cent					
20, p.m.	3.3				2.68	6.70	9.38	6.2	
21, a.m.	9.4	1.018	0.134	8.31	2.44	7.97	10.41	6.6	
21, p.m.	7.7		0.094	8.88	2.26	5.98	8.24	6.6	
22, a.m.	11.1	1.015	0.101	8.49	2.58	6.15	8.73	6.4	
22, p.m.	10.8	1.012	0.105	7.24	2.92	5.41	8.33	6.2	
23, a.m.	12.0	1.013	0.102	6.14	3.02	4.47	7.49	6.4	Operation 10.30 a.m. No water.
23, p.m.	6.9		0.115	2.18	−0.69	1.79	1.10	7.8	
24, a.m.	13.3	1.014	0.110	6.48	1.46	5.10	6.56	6.8+	Quiet, no tremors.
24, p.m.	25.6	1.006	0.113	6.94	0.64	5.62	6.26	6.8+	Constant twitching of eyelid.
25, a.m.	16.0	1.010	0.118	8.89	1.60	7.50	9.10	6.8	Fine general tremors.
25, p.m.	28.8	1.005	0.148	6.66	0.94	7.04	7.98	6.8	" " "
26, a.m.	12.8	1.010	0.105	6.78	1.18	5.10	6.28	6.8	" " "
26, p.m.	15.7	1.007	0.105	6.23	0.87	4.70	5.57	7.0	" " "
27, a.m.	7.6	1.017	0.098	7.95	1.49	5.54	7.03	6.8	Tremors more pronounced.
27, p.m.	4.4		0.124	5.79	1.75	5.00	6.75	6.8+	Quiet, sick, tremors. 7.00–8.00 p.m. 20 cc. $CaCl_2$ solution injected.
28, a.m.	19.7	1.009	0.109	7.46	1.22	5.80	7.02	6.8	Faint tremors.
28, p.m.		1.007						6.6+	Mild tremors.
29, a.m.	15.8	1.009	0.114	11.88	2.01	9.55	11.56	6.6	" "
[illegible]	14.3	1.013	0.139	10.71	0.59	9.49	11.04	6.8	Acute tetany. Died 11.00

TABLE V.

Experiment 99. Female Dog. Weight 10 Kg. Posterior Half of Left Thyroid Retained. 150 Cc. Water per Os Twice Daily except Where Noted.

Average Hourly Excretion.

Date.	Volume.	Specific gravity.	Acid.	NH_3	Total acid.	P_H	
			cc. of 0.1 N solution.				
Mar.	cc.						
10, a.m.						6.0	Fed a.m.
10, p.m.						7.0	Operation 4.00 p.m.
11, a.m.	10.7	1.042	3.05	15.21	18.26	6.2	Quiet, no tremors.
11, p.m.	19.1	1.016	2.39	17.17	19.56	6.0	
12, a.m.	11.7	1.023	2.82	11.38	14.20	6.0	Shivering.
12, p.m.	22.2	1.009	1.44	8.66	10.10	6.2	Faint general tremors.
13, a.m. (1)	9.1	1.007	1.22	6.01	7.23	7.0	(1) Night specimen.
							(2) Catheterized specimen.
13, a.m. (2)						6.2	Faint general tremors.
13, p.m.	9.4	1.015	1.61	5.80	7.41	6.4	" " "
14, a.m.	9.0		0.30	3.57	3.87	7.0	No tremors, asthenic.
14, p.m.	14.7	1.010	1.61	5.15	6.76	6.4	" " "
15, a.m.	12.8	1.013	2.38	7.41	9.79	6.2	No tremors. Operation 3.30 p.m. Remaining thyroid tissue removed.
15, p.m.	13.9	1.006	1.73	4.16	5.89	6.2	No water.
16, a.m.	4.5	1.029	4.66	4.80	9.46	6.2	Mild tremors.
16, p.m.	11.0	1.017	1.05	7.39	8.44	6.8	Violent tetany 7.30 p.m.
17, a.m.	5.5	1.050	4.58	7.58	12.16	6.4	Tetany. No water.
17, p.m.						6.0	Injected $CaCl_2$ solution 10 cc., 11.00 a.m. 8 cc., 12.35 p.m. Fine tremors 6.00 p.m.

TABLE V—*Continued.*

Date.	Volume.	Specific gravity.	Acid.	NH_3	Total acid.	P_H	
			cc of 0 1 N solution.				
Mar.	cc.						
18, a.m.	5.5	1.031	1.99	7.68	9.67	6.0	Fine tremors.
18, p.m.	5.4	1.042	1.31	6.59	7.90	6.6	Tremors more marked.
19, a.m.	13.1	1.022	3.75	7.94	11.69	6.4	Tremors. Injected 5 cc. $CaCl_2$ solution. Improvement.
19, p.m.	19.3	1.009	1.93	7.34	9.27	6.8	No tremors.
20, a.m.						6.4	Mild tremors. Weak. Ataxic.
20, p.m.	11.1		1.45	5.16	6.61	6.8	" " " "
21, a.m.	7.5		2.54	6.10	8.64	6.4	" " " "
21, p.m.	7.6		2.79	6.85	9.64	6.4	" " " "
22, a.m.	9.8		3.20	7.71	10.91	6.4	Constant tremors. Apathetic. Fed milk and calcium lactate. Died Mar. 27.

Average Hourly Excretion.

Date.	Volume.	Specific gravity.	Total N.	$\frac{NH_3\text{-}N}{Total\ N}$	Acid.	NH_3	Total acid.	P_H	
					cc. of 0 1 N solution.				
Apr.	cc.		*gm.*	*per cent*					
27	5.6	1.033	0.136	2.98	3.17	2.90	6.07	6.0	Fed regular diet.
28, a.m.	10.0	1.030	0.258	3.60	3.12	6.58	9.68	6.0	Fed small amount of meat.
28, p.m.	13.6	1.033	0.265	4.19	3.12	7.95	11.07	6.0	Operation 2.30 p.m.
29, a.m.	11.8	1.019	0.183	6.05	2.06	7.93	9.99	5.5	Fine tremors.
29, p.m.	14.9	1.013	0.171	7.85	2.31	9.63	11.94	5.6	Mild acute tetany in a.m. 150 cc. water *per os*.
30, a.m.	15.9	1.016	0.166	7.07	2.62	8.40	11.02	5.6	Acute tetany.
30, p.m.	10.8	1.030	0.239	6.00	4.21	10.25	14.46	6.0	Quiet. Acute attack when catheterized. 100 cc. water *per os*.
May									
1, a.m.	26.6	1.015	0.275	7.07	7.54	13.94	21.48	5.8	*Acute tetany. Injected 125 cc. 0.5 per cent HCl in 0.4 per cent NaCl solution.
1, p.m.	36.9	1.011	0.318	6.65	10.14	15.10	25.25	6.0	**Faint tremors. 0.5 gm. HCl in 150 cc. water *per os*.
2, a.m.	15.9	1.026	0.162	12.91	11.51	14.93	26.44	5.6	***No tremors. Bright. Acid as above.
2, p.m.	10.7	1.017	0.160	7.10	2.83	8.16	10.99	5.5	" " " "
3, a.m.	12.0	1.018	0.134	7.61	3.12	7.25	10.37	5.4	" " " " No tremors until 6th. Fed. Tetany at intervals. Died on 24th.

*Albumin in urine.

ON THE RÔLE OF ELECTROLYTES IN THE DIFFUSION OF ACID INTO THE EGG OF FUNDULUS.

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I.

The experiments of Loeb and Cattell[1] on the diffusion of potassium into or from the egg of *Fundulus* have shown that this process is influenced by the anions in a way suggestive of the Hofmeister series and of Hardy's valency rule. It was of interest to find out whether other cations besides K showed a similar behavior. The writer tested the diffusion of acetic acid into the egg. It was found that this process is retarded by the anions in the same but still more pronounced way than was true for the diffusion of K; but that in addition the counteracting effect of the cation is equally marked and increases also with increasing valency.

The acetic acid was used in concentrations of M/500 (1 cc. M/10 acetic acid in 50 cc. solution). The criterion for the toxic action of the acid was the standstill of the heart of the embryo inside the egg. The eggs used were always over four days old. Soon after the standstill of the heart the embryo and yolk sac underwent coagulation which betrayed itself by the white opaqueness. It was found that the time in which the hearts stopped beating was much shorter when the acid was added to distilled water than when it was added to salt solutions. We will first show that the antagonistic effect of the salt solution increases, within certain limits, with its concentration. Table I may serve as an example. The acid was M/500 acetic acid, the salt was NaCl. Ten eggs were put into each solution.

[1] Loeb, J., *Proc. Nat. Acad. Sc.*, 1915, i, 473. Loeb, J., and Cattell, McK., *Jour. Biol. Chem.*, 1915, xxiii, 41.

TABLE I.

After	Number of embryos with heart beat in 1 cc. M/10 acetic acid in 50 cc.					
	H_2O	M/64	M/32	M/16	M/8	M/4 Na
hrs.						
4	3	0	0	4	10	10
5¼	0	0	0	1	8	10
6¼	0	0	0	0	8	10
9	0	0	0	0	3	8
20	0	0	0	0	0	0

It is obvious that the M/4 NaCl retards the velocity with which the embryos are killed in the acid more efficiently than do the weaker concentrations of NaCl. Very weak salt solutions like M/64 or M/32 may possibly be more harmful than distilled water. An analogue to such a possibility was found in the earlier experiments of Loeb and Wasteneys on the counteraction of the poisonous effects of KCl by NaCl.[2] We will show in Table II that the protective action of salts is a distinct function of the nature and valency of the anion. Each solution contained ten eggs.

TABLE II.

After	Number of embryos with heart beat in 1 cc. M/10 acetic acid in 50 cc.								
	H_2O	M/8 NaCl	M/8 NaBr	M/8 NaI	M/8 $NaNO_3$	M/8 Na acetate	M/8 NaSCN	M/8 Na_2SO_4	M/8 Na tartrate
hrs.									
5½	0	9	10	0	1	10	10	10	10
7½	0	0	1	0	0	10	10	10	10
10½	0	0	0	0	0	10	10	10	10
24	0	0	0	0	0	10	10	10	10
48						8	10	10	10
96						7	10	7	10

It would be difficult to find a more striking demonstration of the rôle of the anion in the counteraction of the toxic action of the acid, the organic anions and the bivalent ones being much

[2] Loeb, J., and Wasteneys, H., *Biochem. Ztschr.*, 1911, xxxii, 155.

more efficient than the inorganic univalent anions Cl, Br, I, and NO_3. The order of efficiency from the weakest to the strongest antagonist is

I, NO_3 < Cl, Br < acetate, SO_4 < SCN, tartrate.

The result is similar to the antagonization of potassium poisoning, except that SCN is much more active in this case than in the case of potassium.

The same order in the relative efficiency of the anion was also found in the case of other salts, *e.g.*, NH_4 and Li, as shown in Table III.

TABLE III.

After	Number of beating hearts in 1 cc. M/10 acetic acid in 50 cc.					
	H_2O	M/8 NH_4Cl	M/8 NH_4NO_3	M/8 NH_4 acetate	M/8 $(NH_4)_2SO_4$	
hrs.						
3	5	9	9	10	9	
8½	0	0	0	10	8	
24	0	0	0	10	5	
48	0	0	0	9	2	
	H_2O	M/8 LiCl	M/8 LiBr	M/8 LiI	M/8 Li acetate	M/8 Li_2SO_4
6½	5	10	9	0	10	10
10½	0	5	2	0	10	10
24	0	0	0	0	8	10
48	0	0	0	0	8	2
96	0	0	0	0	7	1

Acetate and sulphate are again much more efficient antagonists acid than Cl, Br, I, and NO_3. I is the most inefficient of all ions. M/8 $MgSO_4$ was also much more efficient than M/8 $MgCl_2$.

While these results agree in general with those on the diffusion K through the membrane of the egg, the following observations iffer from those made in the case of K. We had seen that $MgCl_2$ counteracted the action of potassium less than NaCl.

In the case of the prevention of the action of the acid it is just the reverse. The antagonistic action of the salts with bivalent cations is very much greater than that with univalent cations. This is indicated by Table IV. Ten eggs with embryos with beating hearts were put into each solution.

TABLE IV.

After	Number of embryos with beating hearts in 1 cc. M/10 acetic acid in 50 cc.									
	H_2O	M/8 LiCl	M/8 NaCl	M/8 KCl	M/8 RbCl	M/8 CsCl	M/8 $MgCl_2$	M/8 $CaCl_2$	M/8 $SrCl_2$	M/8 $BaCl_2$
hrs.										
4½	0	10	10	3	4	5	10	10	10	6
8½	0	1	2	3	2	2	8	10	10	5
24	0	0	0	0	0	0	3	10	10	0
72							1	10	10	0
96							1	10	10	0

$CaCl_2$, $SrCl_2$, and to some extent $MgCl_2$, are much stronger antagonists to acid than were the chlorides of monovalent metals. The hearts stopped beating in KCl, RbCl, and CsCl more rapidly than in LiCl and NaCl, presumably because the action of K, Rb, and Cs on the heart beat was superposed on that of the acid. The difference in the efficiency of M/8 NaCl and M/8 $CaCl_2$ can not be attributed to the higher concentration of Cl ions in the $CaCl_2$ solution since we have seen that the difference in the action of M/8 and M/4 NaCl (Table I) is very much less than that between M/8 NaCl and M/8 $CaCl_2$.

II.

Loeb and Cattell have mentioned that the presence of acid retards the diffusion of potassium into the egg of *Fundulus*. We can show that the same is true for the diffusion of Ca into the egg. In a previous paper we have shown that a 3/16 M $CaCl_2$ solution kills the eggs of *Fundulus* in a couple of days or less. It can easily be shown that acid prevents or retards this effect. It is possible to use a comparatively high concentration of acid in this case, since the $CaCl_2$ renders the acid rather harmless.

500 acetic acid and M/5,000 HCl acted best. Bases had no ch action. It is not advisable to give figures, since the writer ends to continue these experiments.

III. THEORETICAL REMARKS.

It is obvious that in order to kill the embryo the acid must dife through the membrane of the egg and the question arises ether the salt in the outside solution inhibits or retards this fusion; or whether it diffuses with the acid into the egg and events the injurious action of the acid upon the embryo inside e membrane.

Loeb and Wasteneys have shown that salts inhibit also the tion of acid upon the adult fish, but that this action is pracally restricted to chlorides.[3] Tartrates or rhodanates are o toxic to be useful for this purpose. This would indicate that our recent experiments the antagonistic action of the salt must ve consisted in retarding or preventing the diffusion of enough id into the egg to injure the embryo, since we found SCN and rtrate very efficient in the prevention of acid poisoning of the mbryo.

In their earlier experiments on the inhibition of the action of id on the fish Loeb and Wasteneys have shown that in a pure id solution the fish dies very rapidly, obviously through suffotion, the gills becoming unfit for respiration. This action is nibited or retarded by salts. A titration of the acidity of the lution showed no noticeable difference in the absorption of the id in the presence or absence of salts. This seems to contradict our conclusion that in the embryo the action of the salt nsists in its influence on the diffusion of the acid through the embrane. But it is obvious that the two cases differ in this, at the action of the acid on the surface of the egg membrane es not injure the embryo directly, while the action on the surce is decisive in the case of the gills.

We must apparently discriminate between the action of two rtions of the acid, one combining with a colloid of the membane of the egg or cell and forming a salt, and the other portion

[3] Loeb and Wasteneys, *Biochem. Ztschr.*, 1911, xxxiii, 489; 1912, xxxix, 7.

diffusing into the egg and killing the embryo. In the case o the adult fish it is obviously the first portion acting on the sur face of the gill leaves which kills the fish by suffocation. In th case of the egg it is the second portion which kills the embryo When tartrates or SCN inhibit the action of acid on the embry it is obviously the effect of the second portion which is inhibited It is possible that the process of the diffusion of acid is the sam as that discussed in the case of potassium and on this suppositio the action of the anion is intelligible. It would be of importanc to see whether the poisonous action of the acid on the embry is also reversible, at least in its initial stage, and whether th reversibility resembles that of the reversibility of the potassium poisoning reported in the paper by Loeb and Cattell. It may b difficult to decide this question. We must also consider th possible influence of the water absorbed by the membrane on th rate of diffusion of acid into the egg. The writer had shown lon ago that the muscle absorbs water under the influence of acid and that this process is inhibited by salts.[5] It is possible that th diffusion of acid through the membrane is facilitated by the swell ing of the membrane by acid, and that the antagonistic influ ence of the salt might be due to the antagonization of the swelling

SUMMARY OF RESULTS.

1. It is shown that salts inhibit the toxic action of acids upo the embryo of *Fundulus*.

2. This inhibiting action of the salts is a function of the anio as well as the cation. Rhodanates, acetates, sulphates, an tartrates inhibit very strongly, chlorides, bromides, and nitrate much less, and iodides least of all. The bivalent cations Ca an Sr, and to a smaller degree Mg, also inhibit more strongly tha the univalent cations.

3. Since tartrates and rhodanates are much too toxic to be o use in inhibiting the antagonistic action of acids upon the adult fish we must conclude that the antagonistic action of the anions in our experiments consisted in retarding the rate of diffusion of the acid through the membrane.

[4] Loeb, *Arch. f. d. ges. Physiol.*, 1898, lxix, 1; 1898, lxxi, 457.
[5] Loeb, *ibid.*, 1899, lxxv, 303.

THE RELATION BETWEEN THE CONFIGURATION AND ROTATION OF EPIMERIC MONOCARBOXYLIC SUGAR ACIDS.

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Hudson[1] was the first to discover certain relationships between the configuration and the rotatory power of sugars, glucosides, and the lactones of sugar acids. By so doing he also has brought telling evidence in support of the principle of optical superposition, at least for the sugars and sugar derivatives. Accepting the correctness of the principle one may apply it to the differentiation of the monocarboxylic acids of two epimeric sugars in the following way.

Two epimeric acids differ only by the configuration of their α-carbon atoms. These are antipodes. Accepting the magnitude of the optical rotation of this carbon atom as equal to A and that of the sum of the other asymmetric carbon atoms as equal to B, the rotation of one acid is $+A+B=M$ and that of the epimeric form is $-A+B=N$ (M and N being the empirical values). Hence, $B = \frac{M+N}{2}$, $+A = \frac{2M-M-N}{2}$ or $\frac{M-N}{2}$, and $-A = \frac{2N-M-N}{2}$ or $-\left(\frac{M-N}{2}\right)$.

However, the free acids as a rule are unstable in aqueous solution, passing into their lactones, and therefore the observations will have to be made on their salts. The present literature presents only few data regarding the optical activity of salts of epimeric acids, but in every instance when the salts of the same base of two epimeric acids have been obtained it was found that those acids in which the hydroxyl of the α-carbon atom had the same

[1] Hudson, C. S., *Jour. Am. Chem. Soc.*, 1909, xxxi, 66; 1910, xxxii, 338; 1911, xxxiii, 405. Anderson, E., *ibid.*, 1911, xxxiii, 1510; 1912, xxxiv, 51.

position as in *d*-gluconic acid, the calculated value for the rotation of the α-carbon atom had the sign *plus;* and for those having the configuration of the hydroxyl of the α-carbon atom corresponding to *d*-mannonic acid the sign was *minus.* Hence this method may be employed to determine the configuration of sugar acids when chemical methods are for some reason or other not applicable. It is hoped that the principle will be found of service for determining the structure of the α-hexosamines.

The following data are taken from the work of Nef.[2]

Acid.	Formula.	Derivative.	$[\alpha]_D$	Calculated rotation of the α-carbon atom.
d-Gluconic.....	$CH_2OH\frac{H\ H\ OH\ H}{OH\ OH\ H\ OH}COOH$	Calcium salt	+10.5	+9.0
d-Mannonic....	$CH_2OH\frac{H\ H\ OH\ OH}{OH\ OH\ H\ H}COOH$	Calcium salt	−7.5	−9.0
d-Gluconic.....	$CH_2OH\frac{H\ H\ OH\ H}{OH\ OH\ H\ OH}COOH$	Strychnine salt	−18.76	+3.9
d-Mannonic....	$CH_2OH\frac{H\ H\ OH\ OH}{OH\ OH\ H\ H}COOH$	Strychnine salt	−26.7	−3.
d-Gulonic*.....	$CH_2OH\frac{H\ OH\ H\ H}{OH\ H\ OH\ OH}COOH$	Phenylhydrazide	+13.4	+13.
d-Idonic*.....	$CH_2OH\frac{H\ OH\ H\ OH}{OH\ H\ OH\ H}COOH$	Phenylhydrazide	−12.42	−12.
d-Gulonic.....	$CH_2OH\frac{H\ OH\ H\ H}{OH\ H\ OH\ OH}COOH$	Strychnine salt	−17.24	+4
d-Idonic.....	$CH_2OH\frac{H\ OH\ H\ OH}{OH\ H\ OH\ H}COOH$	Strychnine salt	−26.00	−4
d-Xylonic*.....	$CH_2OH\frac{H\ OH\ H}{OH\ H\ OH}COOH$	Brucine salt	−18.70	+4
d-Lyxonic.....	$CH_2OH\frac{H\ OH\ OH}{OH\ H\ H}COOH$	Brucine salt	−27.57	−4

* Rosanoff's nomenclature.

Dr. G. M. Meyer and the writer are engaged in extending the study to a larger number of salts of the sugar acids.

[2] Nef, J. U., *Ann. d. Chem.*, 1914, ccccliii, 204.

THE EFFECT OF INGESTED PURINES ON THE URIC ACID CONTENT OF THE BLOOD.

By W. DENIS.

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It has long been assumed that ingested purines have a marked effect on the uric acid content of the blood, a belief which has risen no doubt from our knowledge of the influence of high purine diets on the amount of uric acid excreted in the urine, but which has but small foundation as regards experimental work on the blood itself. Substantially the only experiments on this subject are those of Brugsch and Schittenhelm.[1] The work of these investigators is, however, of an essentially qualitative nature, and so far as I am aware no quantitative experiments dealing with this problem have as yet been published.

A knowledge of the influence of ingested purines on the uric acid content of the blood is desirable because of the light it promises to throw on the general problem of endogenous versus exogenous purine metabolism; and also because in view of the possible value in clinical diagnosis of uric acid determinations in the blood[2] it has become necessary to know as accurately as possible the influence of therapeutic and dietetic measures on this factor.

The experiments recorded below were made with adult patients in the medical and surgical wards of the Massachusetts General Hospital. The subjects used may be subdivided into the following classes: First (Table I), those designated as "normal;" men and women selected from the surgical services who were normal except for the surgical conditions (hernia and fractures) for the relief of which they had entered the hospital. Experimental work on the hernia cases was begun not less than one week after

[1] Brugsch, T., and Schittenhelm, A., *Ztschr. f. exper. Path. u. Therap.*, 1907, iv, 440.

[2] Folin, O., and Denis, W., *Arch. Int. Med.*, 1915, xvi, 33.

operation, and in the fracture cases three to four weeks had elapsed. Second (Table II), nephritic and cardiorenal cases. Third (Table III), persons suffering from various chronic diseases not associated with kidney inefficiency or with fever.

In some cases it was found more convenient on account of the patient's physical condition or former diet to feed first a purine free diet and then one rich in purine, in other cases it was necessary to reverse this procedure. In every case the diet was the same; during the purine free period the food consisted of eggs, bread, milk, cheese, butter, and fruit. During the period of high purine feeding the eggs and a large part of the bread were replaced by a daily ration of 150 grams of calves' liver, 200 grams of roast beef or chicken, and 200 cc. of "concentrated broth" (prepared by boiling down the ordinary hospital soup to one-fifth of its original volume).

Samples of blood were taken at the end of the high purine period and at the end of the period during which a purine free diet was given, care being taken to draw blood only before breakfast so as to obviate any possible effect of a recent meal. Besides the determinations of uric acid and non-protein nitrogen in the blood I have also made daily determinations of the uric acid in the urine.[3]

From the results given in Table I it is evident that in normal individuals it is possible to feed large amounts of purine-containing food without increasing the uric acid content of the blood. It is true that the number of experiments is not large, and also that the experimental material was carefully selected in order to obtain individuals who gave a history of absolute freedom from any joint diseases (to exclude gout) and who were as far as it was possible to ascertain by physical and clinical examinations free from any signs of kidney inefficiency. It is possible that in a large series of less carefully selected "normal" material a few individuals might be found whose tissues and kidneys might be unable to cope with the large amount of purines ingested and who would under the same experimental conditions be found to have an increase in the circulating uric acid.

[3] These determinations were made by the methods of Folin and Denis, *Jour. Biol. Chem.*, 1912–13, xiii, 469; 1911–12, xi, 527; 1913, xiv, 95.

TABLE I.

"Normal" Subjects.

Remarks.	Blood 100 gm.		Urine.	Days.	Diet.
	Non-protein nitrogen.	Uric acid.	Uric acid excretion, daily average.		
	mg.	*mg.*	*gm.*		
Female, 18 yrs. old, weight 50.4 kg., fractured femur	36	2.0	0.40	5	High purine.
	33	2.0	0.18	5	Purine free.
Female, 21 yrs. old, weight 59 kg., fractured femur	32	2.3	0.70	5	High purine.
	30	2.2	0.30	5	Purine free.
Female, 64 yrs. old, weight 68 kg., fractured femur	28	2.2	0.68	5	High purine.
	25	2.1	0.41	5	Purine free.
Male, 21 yrs. old, weight 77.4 kg., fractured femur	34	1.8	1.12	5	High purine.
	32	1.9	0.48	5	Purine free.
Male, 44 yrs. old, weight 102 kg., convalescent after operation for hernia	28	1.4	0.60	7	Purine free.
	30	1.4	0.98	6	High purine.
Male, 25 yrs. old, weight 58 kg., convalescent after operation for hernia	30	2.0	0.30	8	Purine free.
	30	2.2	0.72	5	High purine.
Male, 28 yrs. old, weight 67 kg., fractured femur	28	1.3	0.36	10	Purine free.
	27	1.3	1.16	5	High purine.

ldition to the meat and liver served to the other subjects this also given 225 gm. of thymus per day.

As was to be expected the experiments presented in Table I show that in the case of individuals with impaired kidney functio high purine feeding causes an increase in the uric acid conten of the blood.

The patients used for the experiments presented in Table II suffered from a variety of pathological conditions, and as is t be expected from such heterogeneous material in some cases marked increase in circulating uric acid was observed while i other cases high purine feeding was without effect.

Experiments (unpublished) on normal men have shown tha the protein intake may be increased from an amount sufficien to give 6 to 8 grams of urea in the twenty-four hour urine to tha sufficient to produce a daily urea excretion of 30 to 50 gram without producing an increase of more than 2 to 3 per cent i the circulating urea.

From the results just presented it would seem that the norma kidney reacts towards an excess of uric acid in a way essentiall similar to that in which it conducts itself towards an excess o urea, and is able to excrete the excess of uric acid presented to i when a diet high in purines is fed, thereby keeping the circulatin uric acid at the same level as that obtained when only endogenou uric acid is to be excreted.

When damage to the kidney has occurred (even when this ha not progressed to the point when nitrogen retention is apparen as shown by the non-protein nitrogen values) an accumulation o uric acid takes place in the blood after a short period of purin feeding.

Both from the theoretical and from the practical side it woul of course have been of interest to have extended this series o observations to another class of pathological material and t have included results obtained on persons suffering from gout Absolutely characteristic cases of gout are difficult to secure i conditions suitable for work of this kind, as during the experi mental period it is of course necessary to exclude medication o any kind. As no suitable material of this class has been avail able in the several months during which this work has been car ried on I have been unable to secure any experimental data alon this line. From the practical standpoint, however, the result-

TABLE II.

Renal and Cardiorenal Cases.

Remarks.	Blood 100 gm.		Urine.	Days.	Diet.
	Non-protein nitrogen.	Uric acid.	Uric acid excretion, daily average.		
	mg.	*mg.*	*gm.*		
R. A. 31. Male, 46 yrs. old, weight 76 kg., alcoholic, cardiorenal disease, edema, ascites	42	2.0	0.28	15	Purine free.
	49	4.4	0.42	4	High purine.
H. O. 31. Male, 43 yrs. old, weight 77 kg., hypertension, arteriosclerosis, cardiorenal disease	40	2.0	0.46	8	Purine free.
	46	2.4	0.80	4	High purine.
R. Y. 7. Male, 42 yrs. old, weight 72 kg., aortic disease, cardiac hypertrophy, arteriosclerosis	37	1.3	0.39	8	Purine free.
	48	2.1	0.68	4	High purine.
F. O. 7. Male, 42 yrs. old, weight 62.5 kg., chronic interstitial nephritis, edema, ascites	32	1.8	0.29	8	Purine free.
	39	3.8	0.52	4	High purine.
H. J. 31. Male, 26 yrs. old, weight 54 kg., cardiorenal disease	40	4.1	0.27	5	Purine free.
	54	4.4	0.65	4	High purine.
W. H. 31. Male, 55 yrs. old, weight 79 kg., cardiorenal disease	30	1.8	0.33	10	Purine free.
	42	2.6	0.60	4	High purine.

TABLE II—*Continued.*

	Remarks.	Blood 100 gm.		Urine	Days.	Diet
		Non-protein nitrogen.	Uric acid.	Uric acid excretion, daily average.		
		mg.	*mg.*	*gm.*		
I. G. 31.	Male, 65 yrs. old, weight 61 kg., chronic lead poisoning, arteriosclerosis, chronic interstitial nephritis	66	3.6	0.30	6	Purine f
		80	4.8	0.64	4	High pu
N. E. 16.	Female, 50 yrs. old, weight 54 kg., acute pyelonephrosis of left kidney (right kidney excised 8 months ago)	66	1.8	0.30	10	Purine f
		81	2.6	0.58	5	High pu
R. I. 31.	Male, 64 yrs. old, weight 71 kg., hypertension, cardiorenal disease	32	2.2	0.38	5	Purine f
		35	2.8	0.81	5	High pu

TABLE III.

Remarks.	Blood 100 gm.		Urine	Days.	Diet.
	Ncn - protein nitrogen.	Uric acid.	Uric acid excretion, daily average.		
	mg.	*mg.*	*gm.*		
L. O. I. Male, 32 yrs. old, weight 50 kg., hypertrophic arthritis	28	2.0	0.60	5	High purine.
	27	1.9	0.28	5	Purine free.
F. K. 16. Female, 24 yrs. old, weight 46 kg., gonorrheal arthritis	26	1.8	0.29	5	Purine free.
	30	1.8	0.66	3	High purine.
C. O. 31. Male, 59 yrs. old, weight 50.5 kg., jaundice, probably due to malignant growth of gall-bladder	28	1.2	0.26	4	Purine free.
	28	1.2	0.40	6	High purine.
H. L. 31. Male, 46 yrs. old, weight 77 kg., mitral regurgitation and stenosis	28	1.8	0.78	4	High purine.
	24	1.8	0.39	4	Purine free.
[illegible] K. 31. Male, 34 yrs. old, weight 70 kg., alcoholic, syphilitic myocarditis	31	2.0	0.33	5	Purine free.
	40	2.4	0.69	5	High purine.
M. O. 31. Male, 52 yrs. old, weight 90 kg., cardiac decompensation, auricular fibrillation	35	3.3	0.36	7	Purine free.
	34	3.6	0.98	3	High purine.
G. N. 31. Male, 25 yrs. old, weight 55 kg., chronic colitis	28	2.3	0.64	4	High purine.
	25	1.5	0.28	5	Purine free.

TABLE III—*Continued.*

Remarks.	Blood 100 gm.		Urine.	Days.	Diet.
	Non-protein nitrogen.	Uric acid.	Uric acid excretion, daily aver gs.		
	mg.	*mg.*	*gm.*		
C. A.* Male, 22 yrs. old, weight 78 kg., syphilis	42	3.8	1.20	4	High purine.
	28	1.7	0.30	6	Purine free.
W. Y. 25. Male, 66 yrs. old, weight 85 kg., convalescent after excision of epithelioma of ear	44	2.6	0.90	5	High purine.
	36	1.8	0.43	4	Purine free.
W. D. 7. Male, 47 yrs. old, weight 100 kg., aortic and mitral disease, marked decompensation	30	4.4	0.47	5	Purine free.
	30	4.4	0.80	5	High purine.
L. M. 7. Male, 28 yrs. old, weight 47 kg., lung abscess (convalescent after drainage)	30	2.5	0.59	4	High purine.
	30	2.2	0.28	7	Purine free.
S. L. 7. Male, 21 yrs. old, weight 63 kg., tabes mesenterica	37	1.4	0.31	5	Purine free.
	37	1.7	0.64	4	High purine.

* In addition to the purine-containing foods given the other patients this man received daily 250 gm. of thymus.

already published by Folin and Denis[4] show that in the blood of persons suffering from gout normal uric acid values are not obtained after several days' abstinence from all purine-containing foods.

[4] Folin and Denis, *Arch. Int. Med.*, 1915, xvi, 33.

SUMMARY.

Results are presented showing the effect of purine free and of gh purine diets on the uric acid content of the blood of normal en and of persons suffering from various chronic diseases. In rmal men no increase in the circulating uric acid is produced the ingestion of large quantities of purines. In persons suf-ing from renal insufficiency a more or less marked increase in e uric acid content of the blood is produced by high purine ding. It is therefore concluded that when the determination uric acid in the blood is undertaken as a diagnostic test the istence on a preliminary period during which no purine-con-ning foods are consumed is unnecessary except in cases in which lney insufficiency exists, or perhaps in the case of persons who bitually consume extremely large quantities of purine- contain-g foods.

NOTE ON THE APPARENT CHANGE OF THE OSMOTIC PRESSURE OF CELL CONTENTS WITH THE OSMOTIC PRESSURE OF THE SURROUNDING SOLUTION.

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I.

Loeb and Cattell[1] have shown that if the eggs of *Fundulus* are previously kept for some time in distilled water or in a very weak salt solution, they offer a greater resistance to the poisonous effects of a M/2 solution of KCl than if they are transferred to the M/2 KCl solution from sea water. This experiment is explained on the assumption that between the meshes of the fibers constituting the membrane the distilled water or weak salt solution collects and forms a barrier to the diffusion of the KCl through the membrane. The same authors have shown that if eggs are poisoned with KCl they do not recover, or recover only very slowly, when put into distilled water or sugar solutions, while they do recover when put into solutions of electrolytes. It seemed, therefore, advisable to ascertain whether when *Fundulus* eggs are put from sea water into distilled water or into weak concentrations of salt solutions some of the distilled water or weak salt solution will collect in the meshes of the membrane, replacing the sea water formerly occupying the same space. If this idea is correct, the freezing point of the eggs must change considerably with the concentration of the solution, in which the eggs are previously kept or washed. On the assumption that the membrane is practically impermeable to water and salt in physiologically balanced salt solutions and in distilled water (which was made probable in previous publications by Loeb), the osmotic pressure of the juice pressed out from eggs of *Fundulus* should be the mean

[1] Loeb, J., and Cattell, McK., *Jour. Biol. Chem.*, 1915, xxiii, 41.

of the following two solutions: (1) the contents of the egg, which are constant for the same set of eggs; and (2) the solution absorbed between the meshes of the outer fibrils of the membrane. On the latter liquid would vary with the concentration of the outside medium.

II.

Fertilized eggs of *Fundulus* were kept for a day or longer in distilled water, M/2, or gram molecular sea water. They were then rinsed several times in tap water and put for a few minutes in distilled water. They were then taken out, drained, rubbed gently between two sheets of filter paper to free them from the water adhering to the outside; then mixed with sand, put into canvas, and their juice was pressed out in a Buchner press. Enough eggs were taken to obtain a quantity of juice sufficient for the determination of the freezing point depression. Table I gives the result of some experiments.

TABLE I.

Fertilized Eggs.

Eggs kept for 24 hrs. or more previously in	Then washed in	Δ of egg content.
Distilled water	Distilled water	0.42°
Distilled water	Distilled water	0.47°
M/2 sea water	Distilled water	0.49°
M sea water	Distilled water	0.57°

This table shows the after effect of the solution in which the eggs had been kept previous to the washing, inasmuch as the eggs that had been kept in M sea water had a freezing point depression of 0.57°, while those that had been kept in M/2 sea water had a freezing point depression of 0.49°, and those kept in distilled water had a freezing point depression of 0.42°. They may have been kept longer than one day in distilled water, although we have no record for this.

This table does not allow us to recognize the result of the washing. The influence of the washing is shown in the next set of experiments. Eggs that had been kept in sea water were washed in sea water diluted with different quantities of distilled water

After this they were freed from the water adhering to the outside by rolling them gently between filter paper. The freezing point depression of the water in which the eggs were washed was measured and is given in the second column of Table II. It is obvious that the depression of the freezing point of the juice from the eggs increases with the depression of the freezing point of the wash water and is always higher than that of the eggs of Table I which had been washed with distilled water.

TABLE II.

Eggs previously kept in	Then washed in sea water with freezing point depression of	Δ of egg content.
Sea water.....	0.52°	0.66°
Sea water.....	0.55°	0.68°
Sea water.....	0.67°	0.72°
Sea water.....	0.68°	0.75°
Sea water.....	0.80°	0.77°
Sea water.....	1.04°	0.88°
Sea water.....	1.93°	1.27°

Two facts stand out clearly. First, that the osmotic pressure of the juice of the eggs grows with the concentration of the wash water, and, second, that while the osmotic pressure of the egg contents is above that of the wash water as long as the latter is $\leq$ 0.68°, it falls below it as soon as the Δ of the wash water becomes 0.80° or more. On the reasoning given above, this would indicate that the real freezing point depression of the contents of the egg seems to lie between 0.75° and 0.77°.

In a third group of experiments the eggs were always washed in a mixture of sea water and H_2O, with a freezing point depression of 0.67°, but the eggs had been kept twenty-four hours previous to the experiment in solutions of different concentrations. This experiment was made to make sure that when the eggs are exposed to another solution for a longer time previous to the washing, the brief washing will not necessarily eliminate this solution completely from the meshes inside the membrane.

The influence of the concentration in which the eggs had been kept before washing showed itself, but was not regular. This latter fact can be understood, since slight differences in the

of the following two solutions:
are constant for the same set
between the meshes of the
the latter liquid would v
side medium.

Fertilized eggs of
distilled water, M
then rinsed sever
in distilled wat
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water adher
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eggs wer
determ
the re

will cause corresponding
ch the outside solution with
embrane.
ggs were used in all the experiments rep
We may mention incidentally that we com
notic pressure of the fertilized with that of the u
egg, but found no difference. This observation
of interest in view of the striking differences found by I
man and Runnström on the fertilized and unfertilized egg c
frog.[2] Such differences do not exist in the eggs of *Fundul*

III. THEORETICAL REMARKS.

We have found that the osmotic pressure of the juice pr
out from the egg of *Fundulus* with the aid of a Buchner
varies according to the concentration of the solutions in v
the eggs had been kept previously. This was explained o
assumption that some of the water in which the eggs were w
or kept previous to the washing adhered to the meshes bet
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[2] Backman, E. L., and Runnström, J., *Arch. f. d. ges. Physiol.*, exliv, 287.

TABLE III.

Eggs previously kept in	Washed in diluted sea water of	Δ of egg content.
M/2 sea water	0.67°	0.71°
M/2 sea water	0.67°	0.81°
M sea water	0.67°	0.80°
M sea water	0.67°	0.99°
Distilled water.....	0.67°	0.64°
Distilled water.....	0.67°	0.74°

structure of the membrane will cause corresponding differences in the tenacity with which the outside solution will adhere in the meshes of the membrane.

Only fertilized eggs were used in all the experiments reported in this paper. We may mention incidentally that we compared also the osmotic pressure of the fertilized with that of the unfertilized egg, but found no difference. This observation may be of interest in view of the striking differences found by Backman and Runnström on the fertilized and unfertilized egg of the frog.[2] Such differences do not exist in the eggs of *Fundulus*.

III. THEORETICAL REMARKS.

We have found that the osmotic pressure of the juice pressed out from the egg of *Fundulus* with the aid of a Buchner press varies according to the concentration of the solutions in which the eggs had been kept previously. This was explained on the assumption that some of the water in which the eggs were washed or kept previous to the washing adhered to the meshes between the fibrils of which the membrane is composed. The question may be raised whether this influence of the concentration of the solution in which the eggs had been kept previously may not be explained on the assumption that the egg membrane is completely permeable to water and the substances dissolved in it. This assumption is contradicted by the experiments of Loeb on the floating of eggs and the duration of life of the embryo of

[2] Backman, E. L., and Runnström, J., *Arch. f. d. ges. Physiol.*, 191[illegible] cxliv, 287.

Fundulus in solutions of high concentration.[3] When we put the eggs of *Fundulus* into a solution of 50 cc. of 3 M NaCl + 1 cc. 10/8 M $CaCl_2$ the eggs will float on such a solution, and the embryo will live for three days or longer, while the newly hatched embryos will die in such a solution in a few minutes. Moreover, Loeb has shown that it is impossible to adapt the fish to such high concentrations by gradually raising the osmotic pressure of the solutions.[4] These results and many similar ones are only intelligible on the assumption that the membrane of the egg of *Fundulus* is practically impermeable for water and salt, as long as the eggs are in physiologically balanced solutions, as was the case in the experiments reported in this paper. The results of the experiments reported in this paper also contradict the assumption that the outside solution diffuses into the egg. If this were the case, the osmotic pressure of the eggs in distilled water should become less and less the longer they remain in distilled water, which is not the case. The variations of osmotic pressure observed in our experiments are easily understood on the assumption that the osmotic pressure of the contents of the egg remains unchanged, but that traces of the solution in which the egg had been kept adhere for some time in the meshes of the fibrils forming the outer part of the membrane.

SUMMARY OF RESULTS.

1. It is made probable by experiment that the osmotic pressure of the contents of the egg of *Fundulus* corresponds to a freezing point depression of 0.76°.

2. The osmotic pressure of fertilized and unfertilized eggs of *Fundulus* is practically identical.

3. When the egg is washed or kept in solutions of different concentrations, the osmotic pressure of the juice pressed out of the egg varies somewhat with the outside contents. This is explained on the assumption that the membrane consists of fibrils and that some of the outside solution adheres to the meshes of the outer part of the membrane, without, however, entering into the egg.

[3] Loeb, J., *Science*, 1912, xxxvi, 637; *Biochem. Ztschr.*, 1912, xlvii, 127.
[4] Loeb, *Biochem. Ztschr.*, 1913, liii, 391.

4. This supports the idea, expressed in a previous paper by Loeb and Cattell, that when these eggs are put for some time into distilled water a layer of distilled water is formed inside the membrane which may act as a barrier to the diffusion of potassium into the egg.

THE SALICYLATES. II. METHODS FOR THE QUANTITATIVE RECOVERY OF SALICYL[1] FROM URINE AND OTHER BODY FLUIDS.[2]

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The quantitative recovery of salicyl from tissues and body fluids involves factors of complexity and difficulty not present in foods and simple aqueous solutions. In its passage through the animal organism, the salicyl group is conjugated with glycocoll forming a compound known as salicyluric acid, whose properties (solubility, melting point, volatility, etc.) differ from salicylic acid. In addition, the presence of colloidal and other substances interferes with a smooth and quantitative recovery of salicylic acid. These features will be alluded to in the discussion of the various procedures referred to, and finally the method upon which we have settled as the most practical will be described.

Our object was to devise some practical way by which it would be possible to recover quantitatively the salicyl group from body fluids, such as urine, blood, and feces, after internal administration of salicylates, and which at the same time would not be too time consuming. It is believed that the procedure which is here described can be successfully applied. Some of the more important methods used by previous workers are described in the

[1] The term "salicyl" is used throughout this paper for the sake of brevity to indicate the salicyl group in whatever form of combination it may occur.

[2] The first paper of this series was reprinted from the Annual Report (1914) of the Therapeutic Research Committee of the Council of Pharmacy and Chemistry of the American Medical Association. Title: The Salicylates. A Historical and Critical Review of the Literature.

text and were tried out with varying success. The results obtained with these will be presented first.

Ether Extraction Method.—This is probably the oldest and most commonly practiced procedure. Ether extraction as a quantitative method was first described by Feser and Friedberger.[3] It consists simply in converting a salicyl compound into free salicylic acid and shaking out with ether until the suspected fluid is salicyl-free. Owing to emulsion formation, to the solubility of pigments, fatty and other substances from body fluids and tissues in ether, this method is mainly suited for qualitative purposes. Even by repeated extraction with ether, purification of the product obtained, and very careful manipulation, it is barely possible to approach quantitative results. It requires at least six to eight extractions in order to render 100 cc. of urine of the average patient receiving full therapeutic doses of salicylate salicyl-free. The contaminated residue resulting after the removal of the ether requires treatment with charcoal and at least two to three recrystallizations to obtain the salicylic acid pure. Ethereal solutions of salicylic acid have a tendency to crawl and spread over the sides of an evaporating dish, and this together with repeated handling entails a considerable loss of salicylic acid. Several attempts were made to obtain a quantitative recovery after the direct addition of salicylic acid to urine or water, but unsuccessfully. The following results will serve to illustrate that ethereal extraction of salicylic acid is not quantitative.

1.002 gm. salicylic acid with the aid of alkali was dissolved in 50 cc. alkaline urine, then acidified with dilute HCl and extracted with ether. The total quantity recovered, of a dark yellowish residue, was 1.015 gm. This was redissolved, treated with animal charcoal, and recrystallized twice. The quantity recovered was 1.012 gm., equivalent to 101.3 per cent recovery.

The same procedure was carried out with 0.503 gm. salicylic acid added to 50 cc. of urine. The recovery was 0.486 gm., equivalent to 96.6 per cent.

In the next trial 0.867 gm. salicylic was dissolved in 100 cc. of urine and this was extracted repeatedly, twelve to fifteen times, before salicyl-free. The weight of the total residue left after evaporation of the ether was 1.036 gm. After recrystallization and treatment with charcoal the weight was 0.907 gm., a difference of 0.129 gm., which represented impurities. The recovery amounts to 104.5 per cent.

Even more disappointing results were obtained with other urines. Owing to the uncertainty of the recovery of added known quantities of salicylic acid, and because it is very tedious and time consuming, simple ether extraction was abandoned as unpractical and inaccurate.

Mosso's Method.—Mosso[4] was the first to call attention to the fact that the investigators of his time usually failed to take account of the salicylur- ic

[3] Feser and Friedberger, *Arch. f. wisssensch. u. prakt. Thierheilk.*, 1875, ii.

[4] Mosso, U., *Arch. f. exper. Path. u. Pharmakol.*, 1890, xxvi, 267.

cid excreted in urine, and he pointed out that the results obtained by them ere only partial recoveries of the salicyl radicle ingested. According o Mosso, the solubility of the salicyluric acid is greater in ethyl acetate an in ordinary ether, so that with a mixture of ether and ethyl acetate oth the salicylic and salicyluric acid could be more completely removed an with ether alone.

In detail the quantitative separation as carried out by Mosso consists first removing the mucoid or other substances by precipitation of the rine with neutral lead acetate; the precipitate is removed and washed until alicyl-free. Then the filtrate is treated with ammonia and lead acetate, nd heated. The precipitate, which now contains the salicylates, is re-oved on a filter paper and decomposed with ammonium carbonate or lphuric acid, filtered, and the precipitate washed until salicyl-free. rom the filtrate the salicylic acids are now removed by repeated extrac-on with small quantities of a mixture of ether and ethyl acetate. This quires about six to eight extractions. The solution is allowed to evapo-te spontaneously from a suitable dish and the crystalline residue remain-g is weighed. The whole is then heated on a water bath until the weight comes constant; it is weighed again and the weight of this second residue rresponds to salicyluric acid. The difference between the weights of the o residues corresponds to salicylic acid which had volatilized.

Mosso administered known quantities of salicylate and collected urine r two to three days. His percentage recoveries were 96.8, 98.5, 102.1, d 106.7 per cent. In our hands the following results were obtained by is method: 126.4, 215, and 223 per cent. The objections to this method e the same as for the simple ether extraction procedure.

Sauerland's Method.—This was described by Sauerland[5] in his study the excretion of salicyl after the application of salicylate ointments to e skin. It is carried out as follows: Urine is acidified, saturated with monium sulphate, and extracted with a mixture consisting of three parts petroleum ether and two parts of chloroform. The ether-chloroform tract is then shaken with water containing ferric alum until no violet lor is obtained. The violet watery extracts are combined and com-red in cylinders with a standard of sodium salicylate. Without the monium sulphate, 80 per cent and 100 per cent with 0.5 gm. and 0.5 mg., spectively, added to urine were recovered. With ammonium sulphate, 3 per cent and 95 per cent were recovered with 0.5 and 0.2 mg. quantities, spectively.

A trial of this method was made by the addition of sodium salicylate to rine. The following results were obtained: 80 per cent recovery with a uantity of 0.086 gm., and 107 per cent with a quantity of 0.043 gm. It vas observed that the violet water faded on standing. This is also pointed out by Sauerland. With larger quantities of salicyl, prolonged extraction with water is necessary and the fading becomes a serious factor. The watery extracts also did not possess the typical violet tint of salicyl in

[5] Sauerland, F., *Biochem. Ztschr.*, 1912, xl, 56.

water. The method was abandoned as inaccurate for our purposes, as we anticipated working with highly concentrated urines as well as with dilute ones.

Direct Colorimetric Estimation of the Salicyl in Ether Extracts of Urine.—This has been practiced by a number of investigators. 100 cc. of urine were collected after the administration of 0.568 gm. sodium salicylate. Several aliquot portions (200 cc.) were taken, acidified, and extracted with ether. The ether was allowed to evaporate spontaneously and the residues were dissolved in water, treated with animal charcoal, and filtered. In no case was it possible to obtain filtrates absolutely free from a brown tint. The filtrates now containing the partially purified salicyl residues in solution were diluted to definite volumes and a colorimetric estimation attempted directly with 2 per cent iron and ammonium sulphate (previously boiled and filtered). In one case the urinary residues gave a bluish violet color with a brown precipitate; with other residues a purple-wine color was obtained. It was impossible to match the characteristic violet color of the standard sodium salicylate in water.

Thus far it is seen that salicyl cannot be accurately and conveniently recovered by any of the methods tried out. Direct estimation in urine is certainly impracticable and impossible. This is even more true of other body fluids, such as blood and joint fluids. The governing principle of any method is the recovery of the salicyl in pure form or in aqueous solution free from any disturbing elements, before it can be estimated quantitatively. After considerable experimentation it is believed that this can be successfully accomplished in three steps: (1) hydrolysis of the specimen containing the salicyl; (2) distillation by steam; (3) colorimetric estimation of the distillate.

These procedures are not new. Each has been practiced by workers before, but so far as we are aware their combination has never been applied to urine. Hydrolysis was long ago practiced with hippuric acid, and later with salicyluric acid as a preliminary to its recovery as salicylic acid by ether extraction and gravimetrically. Distillation of salicylic acid from aqueous solutions with the aid of steam is recommended by the Association of Official Agricultural Chemists, and was also used by Cassal. The colorimetric estimation with iron is quite old. Before the method is described, the various steps and other features will be briefly considered, for there is a paucity of data in the literature, and their desirability is important to justify the usage of these procedures.

Hydrolysis.—The object of this is to decompose the conjugated combined salicyl into free salicylic acid which could then be volatilized by distillation with steam. The conjugated salicyl is thought to exist in the form of salicyluric acid, an analogue of hippuric acid, and there are possibly other salicyl compounds. Thus far we have been unable to prepare any salicyluric acid or other such salicyl compounds satisfactorily. It appears that the urines of most individuals will require hydrolysis because we have encountered urines which, after prolonged (two days to two weeks) continuous ether extraction, until the ether extract no longer gave a test for salicyl, yielded additional salicyl on hydrolysis and distillation. Some urines are somewhat more easily and readily extractable with ether, but we have found in almost every instance with such solvents as ether, ethyl acetate, petroleum ether, and chloroform, some unextracted salicyl remaining in the urine. The quantity depends upon the concentration of the salicyl. Thus far we have encountered such quantities as 10, 20, and 30 mg. unextracted with 100 cc. quantities of urines. On the other hand after hydrolysis for very short and long periods, before distillation no additional salicyl has been found by still more hydrolysis or extraction of the distilled residues with ether and ethyl acetate. We have also encountered fractional specimens of urine voided by the same individual which behave somewhat differently as to the amount of salicyl recoverable by hydrolysis; that is, some specimens would appear to require longer hydrolysis, although less salicyl would be present than in a previous specimen. This may be associated with some differences in the excretion of the combined salicyl (salicyluric acid and others) in the same individual at different times of the day. The question of salicyluric acid will be treated in a later paper.

After the examination of a large number of urines it is certain that hydrolysis can be carried on simultaneously with distillation. In other words, it is not necessary to hydrolyze previous to distillation. Distillation of the average urine (100 cc.) requires about an hour (of more concentrated urines somewhat more), and this is sufficient, for exactly the same results are obtained when the urine is previously hydrolyzed (for different periods) and then distilled. Experiments were made in this direction and

data illustrative of the results obtained will be found in T
Previous hydrolysis with hydrochloric and other acids ha
practiced by others mainly with the idea of facilitating ext
of the salicyl by those solvents suited for ordinary salicyli
However, it must be concluded that previous hydrolysis
necessary with the distillation method.

TABLE I.

Period of Hydrolysis and Recovery of Salicyl by Distillation

No. of urine specimen.	Quantity of urine used.	Period of previous hydrolysis.	Quantity of salicylic acid estimated.	Total volume of distillate.	Remarks.
	cc.	*hrs.*	*gm.*	*cc.*	
8-42	100	½	0.0010	500	
8-42a	50	None	0.0005	500	
8-39	50	1	0.006	500	
8-39a	50	None	0.006	500	
B-2	50	8	0.023	1000	
B-7	50	19	0.021	1000	
B-9	50	None	0.024	1000	
U-1	50	None	0.035	500	
U-6	50	4	0.036	500	
U-7	50	4	0.035	500	Hydrolyzed wi acid.
U-9	50	None	None	500	Distilled with distillate gave test for SO_4; ve
8-30	100	4	0.054	500	
8-30a	50	None	0.027	500	
11-4	100	1	0.180	500	
11-4a	100	None	0.180	500	
11-7	50	4	0.366	1000	
11-7a	50	None	0.369	500	

Choice of Hydrolyzing Agent.—Either acids or alkalies,
times boiling the untreated urine alone, will suffice. W
at once rule out alkalies, since it is necessary to acidify
distillation so as to convert the salicylate into the volat

salicylic acid. Volatile mineral acids (HCl or HNO_3) cannot be used because they pass over into the distillate, render it markedly acid, and interfere with the colorimetric estimation. Sulphuric acid cannot be used because it is decomposed as the residue becomes concentrated and passes into the distillate. Phosphoric acid was found to be most satisfactory. About 20 cc. of the syrupy variety (80 per cent) are used, regardless of the quantity of urine. A smaller quantity would suffice so far as hydrolysis is concerned, but does not permit boiling of the concentrated residue, since the quantity is not sufficient to cover the bottom of the flask.

Decomposition of Salicylic Acid by Hydrolysis.—This was considered at the outset improbable, and the data in Tables II and III show that none occurs. The matter was tested out by

TABLE II.

Effect of Time and Temperature of Hydrolysis on Salicylic Acid.

Experiment No.	Periods of hydrolysis.	Quantity of salicylic acid introduced.	Salicylic acid recovered.	
	hrs.	*gm.*	*gm.*	*per cent*
1	4 (Direct flame)	0.0897	0.0899	100
2	4 (Water bath)	0.0898	0.0899	100
3	2 (Direct flame)	0.0987	0.0977	99

Subjecting known quantities of salicylic acid to hydrolysis under the conditions of our method for different periods of time and under different temperatures. Table II contains the data from such an experiment. Other observations made in various ways confirm the contention that no decomposition of salicylic acid occurs during the periods of hydrolysis under the conditions of distillation here used. It is known that prolonged slow distillation of salicylic acid decomposes it into phenol and CO_2. However this is practically insignificant and occurs under entirely different conditions than obtain in our method.

Recovery of Added Salicylic Acid by Distillation with Steam.—Known quantities of salicylic acid were added to urine, which

was then treated with phosphoric acid and water and subj
to distillation with the aid of steam. Methyl salicylate
acetylsalicylic acid added to urine were hydrolyzed with so
hydroxide, the residues concentrated, acid was added, an

TABLE III.

Recovery of Salicylic Acid from Urine by Steam Distillation.

Amount introduced.	Amount recovered.	Recovery.	Volume of distillate.	Remarks.
		*Sodium Salicylate.**		
mg.	*mg.*	*per cent*	*cc.*	
12.5	12.6	100.8	1000	Distillate clear and colorles
12.5	12.5	100.0	625	" " " "
25.2	25.0	99.2	1000	" " " "
25.2	25.0	99.2	1000	" " " "
89.7	89.8	100.0	500	" " " "
89.8	89.9	100.0	500	" " " "
98.7	97.7	99.0	500	" " " "
789.0	808.0	102.4	1000	Distillate colored greenish.
789.0	792.0	100.4	1000	Distillate colorless.
789.0	792.0	100.4	1000	" "
863.0	740.0	85.7	500	Crystals of salicyl plugge tilling bulb. Loss.
863.0	820.0	95.6	1000	Crystals in condenser an tilling bulb. Loss.
863.0	860.0	99.6	2000	Distillation kept up longer
		*Methyl Salicylate.**		
935.0	893.3	95.6	1000	Distillate turbid.
935.0	934.4	99.9	1000	Distillate clear.
935.0	934.4	99.9	1000	" "
		*Acetylsalicylic Acid.**		
778.2	788.2	101.3	1000	Distillate clear.
778.2	788.2	101.3	1000	" "

* Calculated as salicylic acid.

whole subjected to steam distillation. Seven blank urine
tained from different individuals and with different reactions
also subjected to steam distillation in order to exclude the
bility of contamination of the distillate and interference wit

iron reaction owing to the possible presence of phenols and similar products. It is sufficient to say that distillates from these and other blank urines which we have tested on other occasions did not respond with ferric ammonium alum to any appreciable degree in point of color so as to resemble the phenol-iron or salicyl-iron color.[6] The data obtained with the salicyl urines are presented in Table III and indicate an average recovery of 99 per cent. This shows that salicylic acid can be recovered quantitatively by distillation. This is confirmative of Cassal,[7] who used distillation for the recovery of salicylic acid from aqueous solutions. In general not less than 0.005 to 0.01 gram and no more than 1 gram can be accurately and conveniently recovered with the distillation apparatus used. The average urine is within these limits; probably feces and blood are also.

Preliminary Extraction with Alcohol.—This was practiced by Wiley[8] as a step in the removal of the salicylates from urines after evaporation. The procedure has been used by others, and it was also used in some of our earlier work, mainly with the idea of removing gummy colloidal materials which are sometimes a troublesome feature during distillation. However, it has been definitely ascertained that alcohol does not completely extract the salicylate from the gummy urinary residues in spite of repeated extractions. Hydrolysis of the alcohol treated residues showed that they contain salicylic acid.

This was proved with different urines, treated exactly the same, in the following way: The evaporated urine was rubbed with fine sand and extracted eight to ten times repeatedly with small quantities of alcohol (95 per cent) until salicyl-free. The extracted sand residue was allowed to dry and again extracted, 25 cc. of the alcoholic extract were evaporated, and the iron test was applied. If the test was not positive, the same residue was hydrolyzed as described above and then distilled. The salicyl was estimated colorimetrically. The results are presented in Table IV, and indicate that 1 to 8 per cent of salicyl is not extracted by alcohol.

The Iron Salicyl Test.—This consists of the simple application of any ionizable iron salt to the fluid containing the salicylate. A 2 per cent solution of iron and ammonium sulphate previously boiled and filtered

[6] In Mullikin's tables (Identification of Pure Organic Compounds, New York, 1905) is given a list of volatile substances which give a pink to red color with iron.

[7] Cassal, N. C., *Chem. News*, 1910, ci, 289.

[8] Wiley, H. W., *U. S. Dept. of Agriculture, Bureau of Chemistry, Bull.* 84, pt. ii, 483, 1907.

so that the finished product is nearly colorless or possessing at most a green tint is the best. It cannot be applied to body fluids (urine) dir when small quantities of salicyl are present. A pink to violet color re depending upon the concentration of salicyl. Phenol gives a purple but the reaction is not nearly as delicate. The reaction of salicyl iron is due to the presence of the phenolic group.[9]

The delicacy of this very simple test is sufficient for all practical poses in the quantitative estimation of salicyl in body fluids. Acco to Sherman[10] the delicacy is about 1:1,000,000 when 25 cc. of the sol are used. This has been repeatedly confirmed by us.

The Jorissen Test for Salicyl.—This, according to Sherman,[11] is delicate than the iron test, the delicacy amounting to about 1:3,00 and the color produced is permanent. The test is performed as fol Bring the solution to be tested into a test-tube, add four to five dro 10 per cent sodium nitrite, four to five drops of 50 per cent acetic

TABLE IV.

Extraction of Salicylate by Alcohol from Urine.

Quantity of salicyl extracted with alcohol.	Quantity of salicyl left in sand residue.	Loss.
mg.	*mg.*	*per cent*
54.0	4.5	8
53.5	4.8	8
192.0	2.0	1
152.5	2.7	1.7
75.0	3.6	5
80.0	2.6	3.2

and one drop of 1 per cent copper sulphate. Shake after addition of reagent and finally place in a boiling water bath in such a position the test liquid is completely immersed in the boiling water, and all stand for forty-five minutes. Then remove, allow to cool, and ex against a white background, viewing the tube both vertically and ho tally and comparing with a blank test in which the same amounts reagents have been added to water.

Observations were made with this test with the idea of its po application to urine directly for quantitative purposes. Otherw

[9] Hopfgartner, K., *Monatsh. f. Chem.*, 1908, xxix, 689. Pellet, H., *chim. anal. appl.*, 1901, vi, 328; *Jour. Chem. Soc., Abstracts*, 1901, lxxx, 701.

[10] Sherman, H. C., Methods of Organic Analysis, London, 2nd ed 1912, 378–385.

[11] Sherman, *loc. cit.* Jorissen, A, *Bull. de l'Acad. Roy. des sc. Bel* 3rd series, iii, 259.

would have practically no advantage over the iron test, the delicacy of which is quite as good. 15 cc. of urine of a patient taking fairly large doses of salicylate were treated according to the directions with and without the reagent. The specimen which contained the reagent became a deep brown, the other remaining unchanged (light amber). The same urine was first decolorized with animal charcoal and subjected to the test with the reagent. After heating only ten minutes, it became as deep brown as the non-decolorized urine. When solutions of salicylate in water were used with the test, the characteristic red color was produced. The test is inapplicable to salicylate urine directly, since the quality of the color is entirely changed and it was found also that other urines behaved still differently. Decolorization of the urine does not help. To be applicable at all, the test would have to be used with distillates in the same manner as the iron test is used.

As it consumes more time and reagents, and the delicacy is practically no greater, the Jorissen test has no advantages to offer over the iron test for the estimation of salicyl in urine.

In this connection it might be mentioned that the use of Millon's reagent would have the same objection as the Jorissen. According to Sherman and Gross,[12] the delicacy is 1:2,000,000. It is more time consuming than the iron test, requiring forty-five minutes for the reaction to be completed, but the color is more stable.

Accuracy and Limitations of the Colorimetric Estimation.—In our hands the short 50 cc. Nessler tube (10 cm. length) proved more accurate than the Duboscq colorimeter with 5 cm. cups. With the Duboscq instrument it was impossible to estimate accurately quantities of 5 mg. and less of salicylic acid in 500 cc. H_2O. 1 mg. of salicylic acid in 500 cc. of distilled water was easily recognizable in the flask or in the Nessler tube, but with the Duboscq instrument (5 cm. cup) the solution appeared practically colorless. It is possible to recognize 0.02 mg. of salicylic acid in 50 cc. H_2O (10 cm. column of fluid) in a Nessler tube; untrained workers in the laboratory had no difficulty in recognizing the color when 0.05 mg. was present. With very dilute solutions, equivalent to 0.05 to 0.1 mg. of salicylic acid in 500 cc. H_2O, differences of 0.02 to 0.05 mg. can be made out, and with more concentrated solutions differences of 0.1 mg. can be easily recognized.

[12] Sherman, H. C., and Gross, A., *Jour. Ind. and Engin. Chem.*, 1911, iii, 492.

Solutions containing 5 mg. or more are too deeply colored and do not permit of accurate estimations.

It can be concluded that practically no less than 0.02 mg. and no more than 5 mg. can be determined. This range permits differences of 0.02 to 0.1 mg. to be recognized between solutions.

Interference by Phenolic Substances.—With urines this is insignificant. A large number of non-salicyl urines from different individuals were rendered acid, alkaline, and neutral, and distilled with steam. In no case did the distillate give any appreciable color with iron which would indicate the presence of the barest traces of phenol or salicyl. This is somewhat different with feces of certain individuals, especially those with intestinal disorders. Here a trace of phenolic substances can be found in the distillates. Feces (after evaporation to dryness) of normal individuals and of several patients used in an investigation showed no detectable trace of these substances, and wherever salicyl was present the reaction was practically uninfluenced. The iron-phenol reaction is less sensitive than that with salicyl, and the color obtained overshadows that obtained with phenol. Extremely small quantities of salicyl are found in the feces of patients taking salicylate by mouth, owing to the rapid absorption, so that recovery from feces is for all practical biological purposes unimportant. Nevertheless a procedure for this is here included for whatever worth it may have.

With difficultly absorbable compounds, such as methylene-di-salicylic acid or its acetyl derivative, extraction from feces is necessary to gain a proper idea of their distribution. For this purpose the ether extraction method is used and a colorimetric estimation of the purified extract is made, using a standard made from the same product, since the iron test with some of these products gives a different color from ordinary salicylic acid.

The procedures for the quantitative recovery of salicyl may now be described.

Recovery from Urine.—In order to make a complete salicyl estimation, the urine is collected until salicyl-free, when a little of the voided specimen is extracted with ether and tested with iron. This takes from four to five days after a full therapeutic dose of salicylate, as indicated by certain work now in progress in the laboratory. The total urines are then combined into a con-

osite sample and measured. If the urine is very dilute, it can e concentrated by evaporation on a water bath to about 100 cc., king care that it is neutral or slightly alkaline. Ordinarily, there is moderate water ingestion, hydrolysis may be proceeded ith directly. Formerly we always evaporated the urine to a rupy consistency and extracted with alcohol, but it was found at a loss occurs by so doing, and it is not necessary. With actional and other small specimens which are known to con- in moderate or large quantities of salicyl, we proceed directly ith hydrolysis as soon as the urine is collected and measured.

Hydrolysis and Distillation by Steam.—100 cc. of the urine, gether with 20 cc. of syrupy phosphoric acid (85 per cent), e placed into an Erlenmeyer (Jena) flask of about 450 to 500 cc. pacity, gently boiled over a direct flame, and connected with a attachment for conducting steam through its contents and a ndenser. A distilling bulb connects the flask with the condenser hich serves to carry the salicyl-laden vapors into some suitable ceptacle such as a graduated flask. The distillation is kept up ntil one to two drops of the distillate fail to give a pink color ith one to two drops of iron and ammonium sulphate.

Distillation from the start is carried on nearly to dryness over gentle flame or until the concentrated residue begins to foam nd give rise to a fog within the Erlenmeyer flask. When this curs, the distillation is stopped, more water is added to the stilling flask, and the distillation is continued, the iron test eing in the meantime occasionally applied. As soon as a few rops of the distillate fail to give a positive test, 25 cc. are distilled n addition and again the iron test is applied. If no pink color ppears (indicating that less than 1:1,000,000 of salicyl is present), ne distillation is stopped. The distillate should be perfectly ear, practically colorless, and possess a nearly neutral or a very ightly acid reaction to litmus paper. This is then diluted with stilled water to a definite volume.

Colorimetric Estimation.—For this a standard is prepared by solving 0.1159 gram of dry sodium salicylate in 1 liter of dis- ed water, so that 1 cc. of the standard represents 0.0001 gram l mg.) of salicylic acid. The standard gives a pink to violet or with iron and ammonium sulphate, depending upon the con- tration of salicylic acid present, or quantity used, and re-

mains unchanged for about one week under ordinary laboratory conditions.

The estimation is carried out by taking a definite volume (5 to 10 cc.) of the filtrate, adding a little (0.1 to 0.5 cc.) of the 2 per cent iron and ammonium sulphate, diluting to 50 cc., and comparing with a definite quantity of the standard diluted to 50 cc., containing an equal quantity of iron and ammonium sulphate, until the colors match. At first a rough trial is made, the estimation then being repeated at least two times to get the exact quantities of filtrate and standard which match. The matching of colors is conveniently carried out with the standard Nesbitt tube (short or long variety). As an illustration let us suppose that with the first trial, 5 cc. of filtrate give a color somewhat more intense than 1.5 cc. of the standard.

In the second and third trials 5 cc. of filtrate exactly match 1.8 cc. of the standard. The accepted result, therefore, is that 5 cc. of filtrate exactly match 1.8 cc. of standard.

The calculation of the results can be better illustrated by an example: The total volume of a specimen of urine is 2,000 cc. 100 cc. of the urine which when treated according to the method yielded a distillate of 250 cc. 5 cc. of this distillate required 1.8 cc. of the standard to match.

5 cc. of distillate required 1.8 cc. standard (1 cc. = 0.0001 gram salicylic acid) or 0.00018 gram salicylic acid.

250 cc. of distillate = 50 × 0.00018 gram = 0.009 gram salicylic acid or the quantity represented by 100 cc. of urine. In 2,000 cc. of urine there would be 20 × 0.009 = 0.18 gram salicylic acid, total recovered.

Recovery of Free Salicylic Acid from Blood and Joint Fluid.—Use of this is made in connection with certain experimental observations in progress in the laboratory.

1. Ether Extraction.—An aliquot portion (25 to 100 cc.) or the entire quantity of the whole blood or fluid (unclotted) is taken and extracted with small quantities (15 to 25 cc.) of ether by gently rotating the separating funnel (not shaking violently) until two to three drops of the ether extract evaporated on a watch glass fail to give a pink color with a drop of iron and ammonium sulphate. Usually three to four extractions are necessary. Great care must be exercised as only a small quantity of salicyl may be present.

The fractional ether extracts are placed in a glass evaporating dish, and the ether is allowed to evaporate spontaneously. The residue is treated with hot distilled water until entirely dissolved, taking care to rub down the sides of the dish; this is filtered and the filter paper washed until the washings fail to give a pink color with iron and ammonium sulphate. This may be further purified by recrystallization if necessary.

2. Colorimetric Estimation.—The filtrate is then made up to a definite volume (about 50 cc.) and is ready for the colorimetric estimation as described under *Urine.*

Recovery of Total Salicyl from Joint Fluid and Blood.—After the free salicylic acid is extracted, the residual portion from the separatory funnel may be used if the material is limited. Whole blood cannot be distilled directly on account of the presence of a large quantity of protein and pigment which causes much foaming and yields decomposition products when treated with acid. These can be completely removed by treatment with ethyl alcohol, zinc chloride, and heat. Various other procedures were tried for the removal of these, but unsuccessfully.

Boiling salicylate blood, after dilution with saline, resulted in about 31 to 69 per cent recovery because of the retention of the salicylate by the coagulum, and this could not be effectively washed out. Treatment with ten to twenty volumes of either absolute methyl or ethyl alcohol alone gave opaque filtrates, and when these were distilled with acid, the distillates contained much flocculent material, rendering quantitative estimation of the salicyl impossible. The use of such metals as lead and mercury for precipitation of proteins in the presence of salicylate was precluded on account of the formation of insoluble salicylate salts. Zinc chloride, however, does not precipitate salicylate in concentrated or dilute solutions, and this can be used together with alcohol for the removal of coagulable proteins and other materials in a way similar to that of Folin and Denis in their method for non-protein nitrogen. It was found that absolute ethyl alcohol is no more effective than the 95 to 98 per cent, and that it is necessary to heat finally with zinc chloride in order to remove all traces of proteins and other materials and to prevent opacity in the filtrate and distillate. With beef, human, and dog blood methyl alcohol gives a reddish extract, whereas

ethyl alcohol gave a light pea-green, apparently removing less pigment.

The method in detail is as follows: 15 cc. of the blood containing the salicylate are diluted with 98 per cent ethyl alcohol to 150 cc. and shaken vigorously. After standing about an hour the mixture is filtered. The filtrate has a pea-green color. 100 cc. of the filtrate, representing 10 cc. of the blood, are transferred to a beaker and five to six drops of saturated zinc chloride solution added. The beaker is placed on an electric stove and the contents are boiled gently until nearly all the alcohol is driven off. Then a little distilled water (25 cc.) is added and the mixture concentrated to about one-third by boiling, or until the sides of the beaker show the presence of small yellowish oily droplets. Then the whole is filtered and washed with hot water (three or four times) until salicyl-free (by testing with iron alum). The filtrate should be clear and colorless. If it is opaque, it may be refiltered. A little water and phosphoric acid (20 cc.) are added to the filtrate and the whole is distilled and the salicyl estimated colorimetrically in the distillate in the same manner as described under *Urine*.

As an illustration of the results obtained when salicylate is added to beef and dog blood, the following may be cited: With 5 and 6 mg. quantities, 93 and 95 per cent, respectively, were recovered; with 7.5 and 10 mg. quantities, 95 per cent was recovered. Distillation of such small quantities as 5 to 10 mg. from water alone yielded 95 per cent recovery. Thus it is seen that salicyl can be effectively removed from blood by the method here described. Venous blood from patients taking full therapeutic doses of sodium salicylate has shown the presence of about 3 to 6 mg. in 10 cc.

By combining the absolute quantities of free and combined salicyl, the quantity of total salicyl is obtained.

Recovery from Feces.—The moist feces are previously weighed and mixed so as to form a homogeneous mass. An aliquot portion is dried on a water bath so that about 25 to 50 grams of dried material is obtained. This is thoroughly triturated (using a little sand if necessary), transferred to a paper capsule, and extracted in a Soxhlet apparatus with a mixture of equal parts of ether and ethyl acetate until the extract appears colorless. The

removes neutral fat, fatty acids, and some pigment. Ethyl acetate gives a darker extract (removal of more pigment) than either ordinary ether or petroleum ether alone. The ether extract is discarded[13] and the percolation is continued with 98 per cent alcohol until salicyl-free. Considerable dark pigment appears in the alcohol extract. The alcohol is removed by boiling the extract on an electric hot plate and a little (20 cc.) hot distilled water is added. This results in considerable turbidity. The mixture is now treated with strong milk of lime so as to remove soaps and bile pigments. The salicyl is not precipitated as the calcium salt is quite soluble. It is not always possible to remove the last traces of pigment. The whole is now filtered and the filter washed until salicyl-free. The filtrate is acidified with phosphoric acid and the salicyl of the distillate is estimated colorimetrically in the same way as described under *Urine*. The distillate should be clear, colorless, and practically free from cloudiness or white particles. If present, these can be filtered out. After the addition of 10 to 50 mg. of sodium salicylate to feces 90 to 95 per cent recovery is possible. After the administration of sodium salicylate by mouth, the feces, even when collected for several (4) days, contain very little (10 to 60 mg.) salicyl, and sometimes none at all.[14]

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[13] In the case of methyl salicylate, and similar esters, it would be advisable to test the ether extract with iron.

[14] The distillation method would, of course, not apply to non-hydrolyzable salicyl compounds such as methylene-di-salicylic acid. A special method for this has been worked out and described in another paper.

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THE NATURE OF THE DIETARY DEFICIENCIES OF RICE.[1]

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In former papers from this laboratory we have made clear the nature of the dietary deficiencies of the corn kernel and wheat kernel as the sole source of nutriment for growing animals.[2]

In the present communication we present experimental data showing the specific properties of polished and of unpolished rice as a food, and show the supplementary relationship between these and certain purified and naturally occurring foodstuffs. These studies, in addition to extending our knowledge concerning the dietary position of rice, have contributed to our understanding of the factors involved in normal nutrition, especially as regards the unknown accessory constituents of the diet which have received so much attention in recent years in connection with the "deficiency diseases," scurvy and beri-beri.

I. The Supplementary Relationship between Polished Rice and Purified Foodstuffs.

Gibson[3] found that a partial compensation of the deficient mineral salt content of a diet of polished rice did not prevent, though it seemed to delay, the onset of polyneuritis in fowls. This is in harmony with our results obtained with young rats fed polished rice with such salt additions (Lot 308, Chart 1) as,

[1] Published with the permission of the Director of the Wisconsin Experiment Station.

[2] McCollum, E. V., *Jour. Biol. Chem.*, 1914, xix, 323. Hart, E. B., and McCollum, E. V., *ibid.*, 1914, xix, 373. McCollum, E. V., and Davis, M., *ibid.*, 1915, xxi, 179, 615.

[3] Gibson, R. B., *Philippine Jour. Sc., Section B*, 1913, viii, 351.

from experience with other rations, we know eliminate inorganic portion of the ration as a possible factor in the fa of these animals to grow. This point is illustrated by Lot Chart 2, which received a ration derived from milk pow butter fat, and agar-agar, to which inorganic additions made which gave the entire food mixture an inorganic con closely similar to that of polished rice. Such a modificatio this diet in no way interfered with the growth of young ra

Commonly accepted standards regarding the protein req ment during growth would point to the possibility that the fa of young rats to grow on polished rice with its inorganic deficie corrected might perhaps be due to the low protein content of or to its proteins being of poor quality.

To test this point we fed Lot 316 (Chart 3) with polished a salt mixture, together with 5 per cent of ash-free egg albu prepared according to the procedure described by Taylor.[4] adjustment of both the inorganic and protein portions of the mixture produced no effect on the well-being of the animals. failed to make any growth, and died within two months.

The possibility that polished rice carries some toxic prin which causes injury to the nervous system and thereby the sy toms of polyneuritis has been urged by Caspari and Moszkow If this explanation is valid we might anticipate a noticeable provement in rats when they receive a ration like Lot 316 (C 3), except that the quantity of polished rice was reduced n one-half, this being replaced by dextrin. The history of Lo (Chart 4) shows that this modification of the diet had no bene effect.

The addition of the unknown accessory present in butte and certain other fats[6] to a diet of polished rice plus a salt mi (Lot 317, Chart 5) shows clearly that it is not the lack of this stance which accounts for the failure to grow and maint

[4] Taylor, A. E., *University of California Publications, Pathology*, 19 1, 71.

[5] Caspari, W., and Moszkowski, M., *Berl. klin. Wchnschr.*, 1913, l ii, 1515.

[6] McCollum and Davis, *Jour. Biol. Chem.*, 1913, xv, 167; 1914, xix 1915, xxi, 179; *Proc. Soc. Exper. Biol. and Med.*, 1914, xi, 101. Osb T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1913-14, xvi, 424.

good condition. There was no improvement whatever in animals eating rice, salts, and butter fat, over those similarly fed, but without the butter fat.

In harmony with the failure of purified egg albumin to supplement rice and salts (Lot 316, Chart 3) is the lack of benefit seen as the result of adding purified casein to rice, butter fat, and a salt mixture (Lot 329, Chart 6, Period 1). Here again, lowering the rice content of the ration by nearly one-half failed to benefit the animals, and speaks against the idea that rice is inherently toxic (Period 2). The addition of wheat embryo to the extent of 50 per cent of the food mixture (Period 3) led to prompt growth and prolonged life.

Raising the protein content markedly by the addition of casein to the extent of 13.4 per cent in a mixture of polished rice, casein, butter fat, and salts (Lot 324, Chart 7, Period 1) produced no noticeable effect in inducing growth. The appearance of these rats was very miserable. They were rough coated and emaciated. In Period 2, 5 per cent of lactose (Merck) was introduced into the ration, replacing a portion of the rice, with the result that a slow increase in body weight began which continued over a period of four months with some improvement in appearance.

Lot 340 (Chart 8) proved extremely interesting. The ration differed from Lot 329 (Chart 6) only in having 10 per cent of Merck's lactose replace an equivalent amount of polished rice. There was a marked improvement, and slow growth during two to three months, showing that lactose of the purity of the ordinary reagent still carries some substance having a marked influence in promoting growth in a diet of polished rice supplemented with purified protein, salts, and butter fat.

In Lot 309 (Chart 9), whose ration consisted of polished rice supplemented with purified foodstuffs (casein, butter fat, salts, dextrin) but contained 2.6 per cent of commercial lactose of unknown purity, the animals showed decided improvement over rats which received similar additions to rice, but without the lactose. One vigorous animal reached nearly half the normal adult size and maintained this weight until he was five months old. This result is without doubt to be explained by the fact that the lactose was not very pure. By including 10 per cent of Merck's lactose in the ration the vigorous rat just referred

to was kept from losing weight until he was nine months o (Period 2).

Lot 382 (Chart 10) illustrates the fact that certain preparatio of lactose are by no means as efficient as others in promotin growth when added to rations of polished rice supplemented wit casein, salts, and butter fat. This preparation of Merck's lacto did not improve the well-being of the animals as have some othe preparations. Sweet, Corson-White, and Saxon[7] in their studies o diet in relation to tumor growth, have called attention to simila differences in the dietary effects of different samples of lactose.

Lot 351 (Chart 11) obtained all its protein from 71.4 per cen of polished rice. It seems probable that the failure of 20 pe cent of Merck's lactose to induce growth in this instance was th result, in part, of the low protein content of the diet.

The performance of Lot 355 (Chart 12) makes it clear that th effect produced by lactose in certain of the rations previous described, is not due to its containing the same accessory sub stance as is carried by butter fat. This lot was given a ratio which contained 20 per cent of butter fat without any appare benefit from this generous supply of this unknown factor. Th experiments already described force us to accept the conclusio that *there are necessary for normal nutrition during growth tw classes of unknown accessory substances, one soluble in fats and a companying these in the process of isolation of fats from certa foodstuffs, and the other soluble in water, but apparently not in fat* It will be shown later that the water-soluble accessory is also sol ble in alcohol. The latter substance is present in milk and removed from milk sugar only by thorough crystallizatio Stepp has expressed the belief that there is more than a single cla of unknown accessory substances necessary for prolonged mai tenance of an animal, but he employed only solvents for th lipoids in preparing his foodstuffs.

The curves of Lot 326 (Chart 13) are offered in further suppo of our contention that milk sugar of the ordinary purity may contaminated with sufficient of the water-soluble growth-promo ing accessory to cause pronounced increase in body weight animals whose ration is adequate except for this factor.

[7] Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *Jour. Biol. Che* 1915, xxi, 314.

The records of Lot 383 (Chart 14) are in marked contrast to Lots 329, 324, 355, and 326 (Charts 6, 7, 12, 13) all of which make it evident that *purified proteins, fats having the growth-promoting property, and salt mixtures of appropriate composition, cannot adequately supplement polished rice so as to produce a diet which will support growth.* These curves (Lot 383) reveal the fact that *unpolished rice* is so supplemented by additions of purified foodstuffs as to make a food mixture which supports normal growth. In this respect unpolished rice is like whole wheat, which we have previously shown[8] is completely supplemented by casein, salts, and butter fat, so as to produce normal growth, reproduction, and rearing of the young.

II. The Supplementary Relationship between Polished Rice and Certain Naturally Occurring Foodstuffs.

We have as yet made no experiments with isolated rice fats, but a consideration of the curves of Lots 396, 392, and 395, Charts 15, 16, and 17, indicates that the fats of rice do not contain much if any of the fat-soluble accessory essential for growth. Lot 396 (Chart 15) was fed a mixture of rice 58 grams, rice polishings 40 grams, and Ca lactate 2 grams. The curves show that rice polishings in amount sufficient to furnish about 5 per cent fat do not supply enough of this accessory to permit of any growth. From the results of feeding unpolished rice, Lot 383 (Chart 14), it is evident that rice polishings carry the water-soluble accessory; but without the addition of the fat-soluble accessory in the form of butter fat we have not secured growth on rations composed of mixtures of polished rice and rice polishings with appropriate salt additions. (Compare Charts 8 and 9, in the following paper.) Lot 392 (Chart 16) illustrates the fact that there is nothing appreciably toxic in rice polishings, even when fed alone with the addition of a small amount of calcium lactate. This ration carried about 12 per cent of rice fats, and since there was a slight increase in body weight in each case it would appear that there is a very small amount of the fat-soluble accessory present, but that the amount is inadequate.

[8] McCollum and Davis, *Jour. Biol. Chem.*, 1915, xxi, 643.

The nearly normal rate of growth on a mixture of polished rice 82, rice polishings 10, butter fat 5, and a salt mixture 3 per cent (Lot 395, Chart 17) points again, especially in connection with Charts 15 and 16, to the belief that rice polishings, which carry most of the fat from the rice kernel, do not provide the fat-soluble accessory in amount essential for growth. Here again we have definite evidence that rice polishings contain the water-soluble accessory.

Excellent growth with reproduction has been obtained on a monotonous mixture of polished rice 80 and desiccated egg 20 per cent (Lot 311, Chart 18). This shows that the egg contains both the fat- and water-soluble accessories. This result is further confirmed by the fact that egg yolk alone will induce good growth in young rats.[9] Attention should be called to the fact that both whole egg and polished rice are among the natural foodstuffs carrying an excess of potential acidity in their mineral content.

Regarding the minimum amount of egg which must be added to polished rice in order to supply the two classes of accessory substances in amount sufficient for growth, little can be said at present. Chart 19, Lot 323, Period 1, shows that 5 per cent of desiccated egg is not a sufficient quantity for this purpose. (The low protein content of this ration was not the cause of failure to grow. See Chart 33, Lot 381, and Chart 20, Lot 337.) Since in Period 2 these rats received a ration (Ration 312) which carried a mineral content closely similar to polished rice, the inorganic factor is eliminated as a possible cause for failure of the rats to grow in Period 1.

That 5 per cent of desiccated egg does supply enough of the fat-soluble accessory is shown by the curves of Lot 337 (Chart 2[illegible]) where the rice-egg mixture was supplemented by 10 grams of lactose per 100 of ration (Period 2). Growth was secured through the added amount of the water-soluble accessory contained in the lactose. In Lot 323 (Chart 19), therefore, the failure of [illegible] per cent of desiccated egg to induce growth was apparently due to its inadequate content of water-soluble accessory.

The failure of certain combinations of natural foodstuffs to induce growth because of a deficiency of one or both of the acces[illegible]

[9] McCollum, *Am. Jour. Physiol.*, 1909-10, xxv, 127.

ry substances is further illustrated by Lot 354 (Chart 21) hich was fed a mixture of polished rice 82.4, wheat embryo 13.3 :r cent, and a salt mixture 4.3 per cent. No appreciable amount growth could be secured with this ration, the reason being that)t sufficient fat-soluble accessory was present. The same ra- on with 5 per cent of butter fat replacing an equivalent amount rice (Lot 339, Chart 31) induced normal growth and supported production, and certain young from these rats are still thriving 1 this ration.

In a previous paper we have given evidence that the fat-soluble :cessory is carried by wheat embryo.[10] This material contains)out 10 per cent of oil. In Lot 369 (Chart 22) which was fed) per cent of wheat embryo with polished rice and salts there was rnished by the ration enough of the fat-soluble accessory to ıpport growth for a time at somewhat below the normal rate.

Wheat embryo contains a high content of the water-soluble :cessory. This is shown by the curves of Lot 377 (Chart 23). ven 2 per cent of wheat embryo with rice, salts, and butter fat duces a fair amount of growth (Period 1). The rate of growth as limited by the protein content and not by a lack of water- luble accessory, since the addition of 5 per cent of casein in eriod 2 led to much more rapid growth.[11]

Skim milk powder (Merrill-Soule) is also very rich in the water- luble accessory, since in combination with polished rice 2 per nt of milk powder supplies enough of this substance for nearly rmal growth (Lot 378, Chart 24).

When 4 per cent of wheat embryo is combined with polished :e, salts, and butter fat, Lot 360 (Chart 25), the condition and te of growth of the rats were noticeably better than of those ıich received only 2 per cent of the embryo. This may reason- ly be assigned to the slightly higher protein content of the ra- n of the lot which received 4 per cent of wheat embryo addi- n, rather than to the higher content of water-soluble accessory. th these factors may, however, have operated to induce this sult. The improvement of Lot 361 (Chart 26) which was fed er cent milk powder with polished rice, salts, and butter fat e_, received all their water-soluble accessory from 4 per cent of

[10] McCollum and Davis, *Jour. Biol. Chem.*, 1915, xxi, 179.
[11] See also Chart 4, *ibid.*, 1915, xxiii, 231.

skim milk powder), over Lot 378 (Chart 24) is probably to be similarly accounted for. Both in Lots 377 and 378 the protein content was somewhat too low to admit of growth at the maximum rate.

With certain mixtures of polished rice and wheat embryo, supplemented with salt additions and added fat-soluble accessory (in butter fat) very vigorous growth may take place. Lot 350 (Chart 27) which was fed 82.7 per cent of polished rice and 8 per cent of wheat embryo is an illustration. Since we have shown that the fat-soluble accessory is found in the plant kingdom,[12] it is evident that, employing the knowledge which we have at the present time, it should be possible to compound rations strictly of vegetable origin which will induce perfectly normal nutrition. Experiments in this direction will be reported on later.

In order to determine the distribution of the water-soluble accessory in the wheat kernel we tried feeding polished rice with 8 per cent of wheat bran plus salt and butter fat additions (Lot 357, Chart 28). We learned, however, that commercial wheat bran always contains a small amount of the embryo, and since there are still no data showing the efficiency of the proteins of the bran in supplementing the protein of polished rice for growth it is not possible to say definitely whether or not the water-soluble accessory is limited to the embryo. The results of studying the curative power of rice polishings in polyneuritis throw no light on the distribution of the curative agent in the seed, since in removing the bran layer of rice the exposed embryo of the seed is also removed. Rice polishings consist of a mixture of bran and embryo, and it is not improbable that the widespread view that it is the bran layer of rice which contains the curative power may be erroneous. It seems to us that the experience of investigators that doses of extract of rice polishings equivalent to 10 grams of the polishings are necessary to produce noticeable curative effect on fowls, points to the belief that the embryo portion of the polishings is the source of the active principle. Wheat bran 8 per cent is no more efficient than wheat embryo 4 per cent in promoting growth (Lots 357 and 360, Charts 28 and 25). Since 10 grams of wheat embryo added to 500 grams of a ration of rice, salts

[12] McCollum and Davis, *Jour. Biol. Chem.*, 1915, xxi, 179.

casein, and butter fat will induce good growth, it seems highly probable that the growth-promoting substance (water-soluble accessory) is concentrated in the embryo. We are studying this question further.

A high content of polished rice in the diet is not at all injurious to animals, provided the diet contains suitable supplementary additions. Lot 310 (Chart 29) received 85 per cent polished rice with 8 per cent milk powder, salts, and butter fat, and has grown and remained in good condition during eight months. The low protein content of this diet probably accounts for their failure to reach the normal adult size and to reproduce. This view is supported by the behavior of Lot 335 (Chart 30) in which the milk powder was added to the extent of 15 per cent, but the ration was otherwise like the preceding (Lot 310). Here growth and well-being have been good, as is attested by the rapid rate of growth and repeated reproduction, in one case to the third generation. The mortality of the young was somewhat high, a fact for which we have as yet no adequate explanation, but the ration is apparently adequate for growth in the second generation, some of the curves of which are shown (Chart 30). Satisfactory growth we have shown is no criterion that the ration will be adequate for reproduction. When the wheat embryo was increased to 13.3 per cent of the food mixture in a diet of rice 77.4, wheat embryo 13.3, butter fat 5, and salts 4.3 per cent (Lot 339, Chart 31), the addition of the water-soluble accessory and adequate protein supplementing was accomplished and nutrition was close to normal. When one considers that this ration, which carried only about 10 per cent of protein supported normal growth and repeated reproduction in these females, it must be admitted that wheat embryo in the proper amount supplements polished rice very satisfactorily.

III. The Supplementary Relationship Between Certain Extracts of Naturally Occurring Foodstuffs and Polished Rice.

Among all the naturally occurring foodstuffs, the yolk of a boiled egg yields, we believe, the smallest amount of water-soluble organic matter. The proteins are rendered insoluble by coagulation during heating, the fats are not soluble in appreciable degree

in water, and any emulsified fats in the water extract are easily removed by ether. There are no appreciable amounts of carbohydrates. Since egg yolk is highly efficient in inducing growth it should on extraction with water yield the water-soluble accessory contaminated with some inorganic salts and but a slight amount of organic impurities. That such is the case is made clear by the curves of Lot 367 (Chart 32) which was fed Ration 324 (Chart 7), but in addition the water extract of 6.4 grams of dry boiled egg yolk per 100 grams of ration. These rats made vigorous growth with this addition, while the same ration without the extract produced no growth whatever. 200 grams of dry egg yolk extracted with 800 cc. of water in successive small portions yielded after removal of the emulsified fat only 4.5 grams of water-soluble substances, mostly inorganic salts. That this small amount of material rendered 3.12 kg. of ration efficient for growth shows how slight must be the quantity of active principle necessary for normal nutrition. The temporary fall in body weight of these rats after five weeks' feeding with the extract was caused by the employment at that point of an extract made from egg yolk which had undergone some bacterial decomposition owing to the steam having been turned off the drying oven for a time while the yolks were being dried. On substituting a new preparation of extract from untainted yolks growth was at once renewed.

The stimulating effect of water extract of boiled egg yolk on growth is again shown in the records of Lot 381 (Chart 33). In Period 1, during five weeks growth proceeded on a ration of rice 88, butter fat 5, and salts 3 per cent, together with 4 grams of dextrin upon which the water extract of 8 grams of dried boiled egg yolk had been evaporated. All the protein of this ration (5.8 per cent) was furnished by its content of 88 per cent of rice. In the second period the hot water extract of 5 grams wheat embryo per 100 of ration was employed to furnish the water-soluble accessory. With these extracts growth was continued during three months. There was added from wheat embryo 0.03 gram of nitrogen per 100 grams of ration, or about 3.27 per cent of the total nitrogen content of the ration from this source. The preparation and description of the extracts of egg yolk and wheat embryo employed in this paper are described on pp. 193–194.

The ready solubility of the unknown accessory in water is shown by the extremely rapid growth of Lot 401 (Chart 36). This ration differed essentially from that of Lot 324 (Chart 7), which does not support growth, only in containing the water extract of 15.9 grams of wheat embryo per 100 of ration. The water extract added to this ration is much higher than is essential for growth. This is shown by the curves of Lot 385 (Chart 35) whose ration was identical with the preceding one, except that it contained the water extract of only 5 grams of wheat embryo per 100 grams of ration. Even here the growth is somewhat more rapid than the normal expectation.

The growth-promoting accessory hitherto referred to as the water-soluble accessory is soluble in alcohol as well. Lot 399 (Chart 38) illustrates in a striking manner the stimulating action on growth of a small amount of the material extracted from wheat embryo by boiling acidified 90 per cent alcohol. It should be remembered that this ration without water or alcohol extract additions does not support growth. The hot, acidified alcoholic extract of 10 grams of wheat embryo included in each 100 grams of ration, induced growth at a rate much faster than the normal. Only about 1.40 per cent of the nitrogen of the ration was added in the alcoholic extract.

Lot 400 (Chart 39) received a ration of the same character as the preceding, but with the hot acidified alcohol extract of 5 grams of wheat embryo per 100 grams of ration. The growth of these rats was distinctly slower than that of Lot 399 (Chart 38), but still somewhat faster than the normal expectation. Growth appears to be, within certain limits at least, dependent upon the amount of the accessory present.

95 per cent alcohol without the addition of acids readily extracts the water-soluble accessory from wheat embryo. This is shown by the curves of growth of Lot 386 (Chart 40) whose ration contained the alcoholic extract of 10 grams of wheat embryo per 100 of ration. 0.018 gram of nitrogen, equivalent to about 0.6 per cent of the total nitrogen of the diet, was in this ration derived from the wheat embryo extract. The addition of more nitrogen to this ration in the form of pure protein would have no effect in inducing growth.

Acetone likewise extracts to some extent from wheat embryo the active principle contained in the water and alcohol extracts.

Lot 387 (Chart 41) grew on a ration similar to the ones we h employed in the work with other extracts. Acetone is, h ever, not as good a solvent for the active principle as is wate alcohol. Our experience confirms the observation of Step that certain accessories essential for growth are soluble in s degree in acetone.

The studies of the dietary deficiencies of rice which we h described in this paper illustrate a method of procedure wh yields valuable information of a kind which has not hitherto b available, concerning the supplementary relationship betw rice and a number of the proximate constituents of foodstu and between rice and certain naturally occurring food substan Such knowledge when available for a wide variety of foodst must, we believe, be of great value in the formulation of hun dietaries which will promote health. Furthermore, it must p duce far reaching economic improvement in the feeding of fa animals. When we see that a ration carrying as low as 10 per c of protein (Lot 339, Chart 31) and this derived entirely from ve table sources can serve to support vigorous growth when sup mented adequately by one of the growth-promoting fats and inorganic salt mixture, we must realize the great possible sav in the cost of feeding animals when it becomes known just wh the favorable combinations of protein lie. This combination rice and wheat embryo fed without a fat of the growth-promot group would have been pronounced a failure as a diet. Ther presented here convincing evidence that the older practice experimenting with combinations of natural foodstuffs is searching enough in character to reveal any of the fundamen principles of nutrition or to lead to the acquisition of the k of knowledge of the specific properties of our naturally occur foods, which could lay the foundation of a system of feed based upon scientific principles.

SUMMARY OF CONCLUSIONS.

1. Polished rice cannot be supplemented so as to produc ration which will induce growth by the addition of purified p tein, fats which possess the growth-promoting property, and additions.

[13] Stepp, W., *Ztschr. f. Biol.*, 1913, lxii, 405.

2. The inorganic content of polished rice has been closely imitated by suitable additions of salts and free mineral acids in a ration derived from milk powder and dextrin and in one from desiccated egg and dextrin, without causing any loss of growth-promoting power of the food mixture.

3. Polished rice does not exert a toxic effect on animals even when it constitutes as much as 80–90 per cent of the food mixture. Simple mixtures of rice and egg, rice and milk powder, rice and wheat embryo, carrying such a content of rice, have proven perfectly satisfactory for growth and for prolonged well-being.

4. The addition of quantities of wheat embryo or of milk powder as small as 2 per cent of the food mixture, consisting aside from these constituents, of polished rice, casein, salts, and butter fat, furnishes enough of an essential accessory to induce growth.

5. The essential accessory aside from that carried by butter fat is present in water and in alcoholic extracts of wheat embryo and of egg yolk.

6. The accessory substance which is soluble in water and in alcohol is stable to heat. Prolonged boiling does not injure it to a noticeable degree.

7. The amounts of water extract (freed from protein by coagulation) which we have found necessary to supply enough of the water-soluble accessory to induce normal growth, carry nitrogen equivalent to about 1.0 per cent of the total nitrogen of the ration. Amounts of alcoholic extract of wheat embryo carrying as little as 0.6 gram of solids, and 0.0095 gram N = 0.33 per cent of the total nitrogen of the ration suffice to induce normal growth.

8. The water-soluble accessory is not the same one as is furnished by butter fat. 20 per cent of butter fat addition does not induce any growth unless the other accessory is supplied.

9. Polished rice and salts, together with sufficient wheat embryo to supply liberal protein and water-soluble accessory additions, do not support growth. The fat-soluble accessory must likewise be supplied before growth can proceed.

Preparation of Extracts Employed in the Rations.

1. The Cold Water Extract of Wheat Embryo.—400 gm. of wheat embryo were stirred up with 4 liters of water and allowed to stand with occasional stirring for one hour. The solution was then separated from the solids

left undissolved by centrifugation. The solution was then acidified with acetic acid and boiled to coagulate the proteins. The coagulum was filtered off on a paper pulp filter with suction, and evaporated on 200 gm. of dextrin. The dextrin was ground when dry. About 77 gm. of solids were extracted from 400 gm. of embryo.

2. The Hot Water Extract of Wheat Embryo.—The procedure differed from the preceding only in that the water was heated to boiling and the embryo slowly stirred in. Acetic acid was then added to induce coagulation of the proteins. The solution was allowed to cool, then centrifugated, the liquid filtered and evaporated on dextrin as in the case of the cold water extract. About 95 gm. of solids were in this manner extracted from 400 gm. of embryo.

3. 95 Per Cent Alcoholic Extract of Wheat Embryo.—Wheat embryo was extracted for eight hours with 95 per cent alcohol, in a continuous extraction apparatus. The alcohol together with sugars, fats, etc., in the flask, was then placed on dextrin and the solvent was evaporated. About 8 gm. of solids were extracted from each 100 gm. of wheat embryo.

4. Acid Alcoholic Extract of Wheat Embryo.—400 gm. of wheat embryo were treated with 800 cc. of 90 per cent alcohol and 10 cc. of concentrated HCl added. The alcohol was heated to incipient boiling, and filtered with suction on a pulp filter which had been washed with alcohol just before use. The filtrate was placed on 200 gm. of dextrin and neutralized with NaOH. The solvent was then evaporated off.

5. Cold Acetone Extract of Wheat Embryo.—300 gm. of wheat embryo were treated with 400 cc. of acetone and allowed to stand over night. The acetone was removed by pressure, filtered, and evaporated upon dextrin sufficient to prevent stickiness in the product. The hot acetone extract was similarly prepared except that the acetone was heated and removed while hot. About 7 gm. of solids were dissolved from 100 gm. of wheat embryo.

6. Water Extract of Boiled Egg Yolk.—200 gm. of dry boiled egg yolk were ground in a mortar with water (200 cc.) and then the solution was removed by filtration with suction. A paper pulp filter was employed. This was repeated four times. The solution contained some fat in emulsified form, which was removed by shaking with ether. The ether was mechanically separated, the solution again filtered, and evaporated on dextrin. 200 gm. of dry egg yolk yielded about 4.5 to 5 gm. of solids.

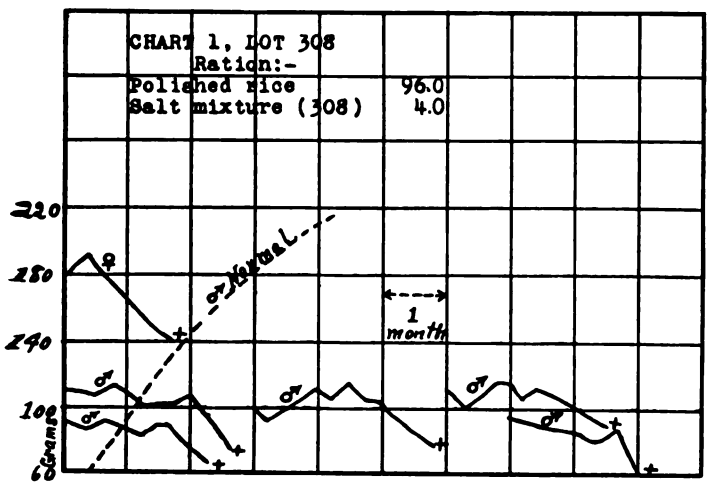


CHART 1. Lot 308. Relation between polished rice and purified foodstuffs. These curves illustrate the fact that polished rice cannot be supplemented so as to induce growth or prolonged maintenance by the addition of a suitable salt mixture alone. That failure of maintenance was not due to the low protein content of the ration, nor to the presence of proteins of a poor character in rice, is proven by the curves of Lot 381, Chart 33, which grew fairly well on a ration, the protein of which was derived solely from polished rice and was appreciably lower than in the ration here employed. We have successfully employed other rations for growth which contained mineral contents closely similar to the above ration (308).

Salt mixture 308:

	gm.
NaCl	5.00
K_2HPO_4	12.10
$CaH_4(PO_4)_2 + H_2O$	2.56
Ca lactate	29.44
Fe citrate	1.00

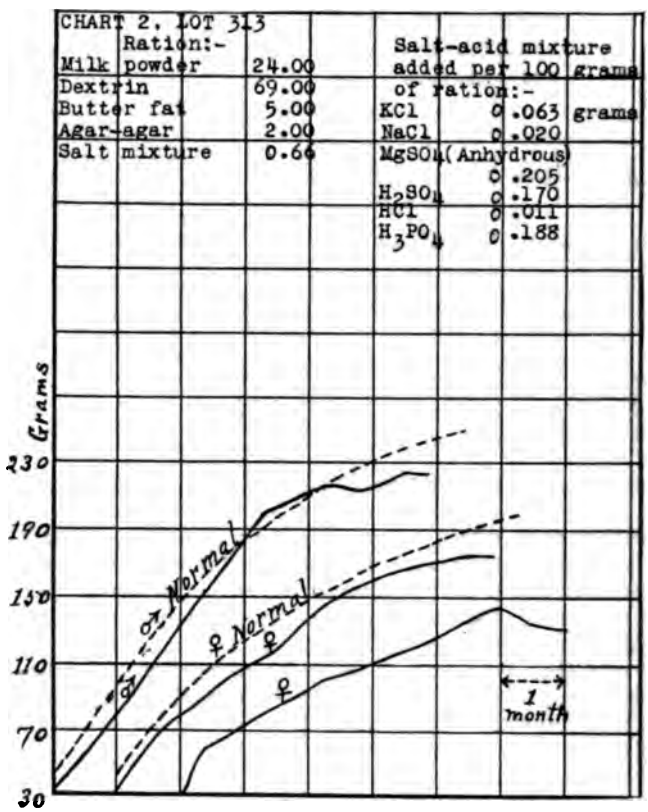


CHART 2. Lot 313. In this ration the mineral content was adjusted by salt and free mineral acid additions so as to approximate closely the mineral content of polished rice. The excellent growth curves make it clear that for growth the mineral content cannot be solely responsible for the failure of animals to grow on a diet of polished rice.

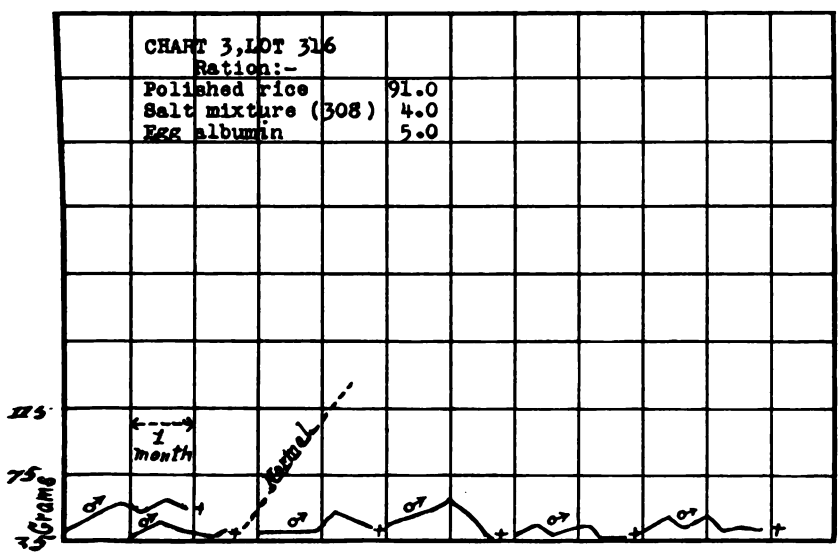


CHART 3. Lot 316. The above curves show that polished rice is not supplemented so as to induce growth or prolonged well-being by correcting the mineral content and the addition of 5 per cent of ash-free egg albumin. It is evident from these curves and Charts 1 and 2 that the deficiency of rice rests in something other than the inorganic or protein factor.

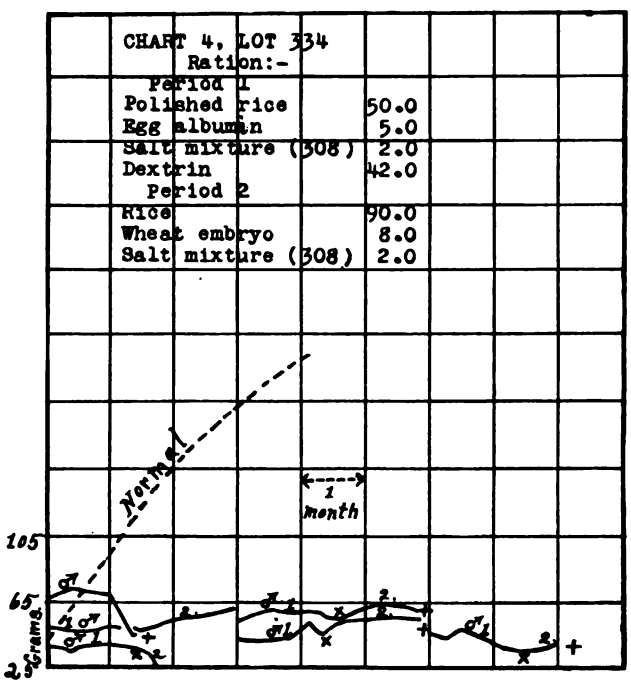


CHART 4. Lot 334. That there is not in rice an inherent toxicity responsible for their failure to grow on high levels is indicated by the performance of the rats whose curves are here shown. The rice was reduced to 50 per cent of the ration instead of 91 per cent as in Lot 316, Chart 3. The condition of the animals was not bettered by the change in the composition of the ration. (See also Lot 338, Chart 9 in the paper following this.)

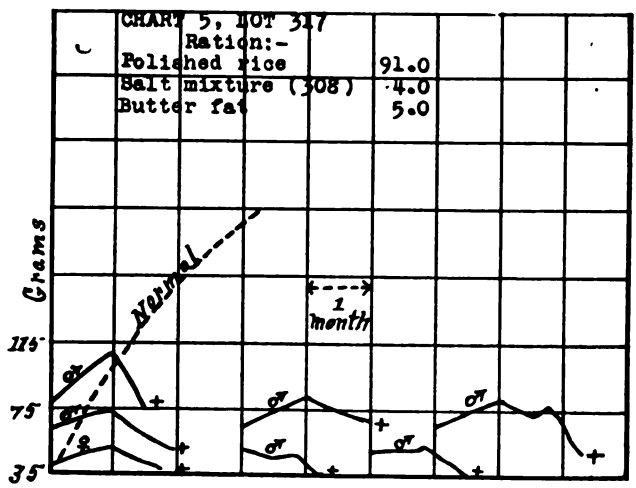


CHART 5. Lot 317. From the above curves it is evident that the failure of rats to grow or be long maintained on a diet of polished rice, a salt mixture, and butter fat, involves some factor other than the lack of the accessory of unknown nature found in certain fats, as butter fat, egg fat, kidney fat, corn, etc., but not in certain others. The protein carried by this ration is adequate for growth at a fairly good rate. (Compare Lot 381, Chart 33.) The mineral content is satisfactory (Lot 381), and the fat possesses the peculiar biological properties which promote growth, yet young rats cannot long maintain their body weight or well-being on this ration.

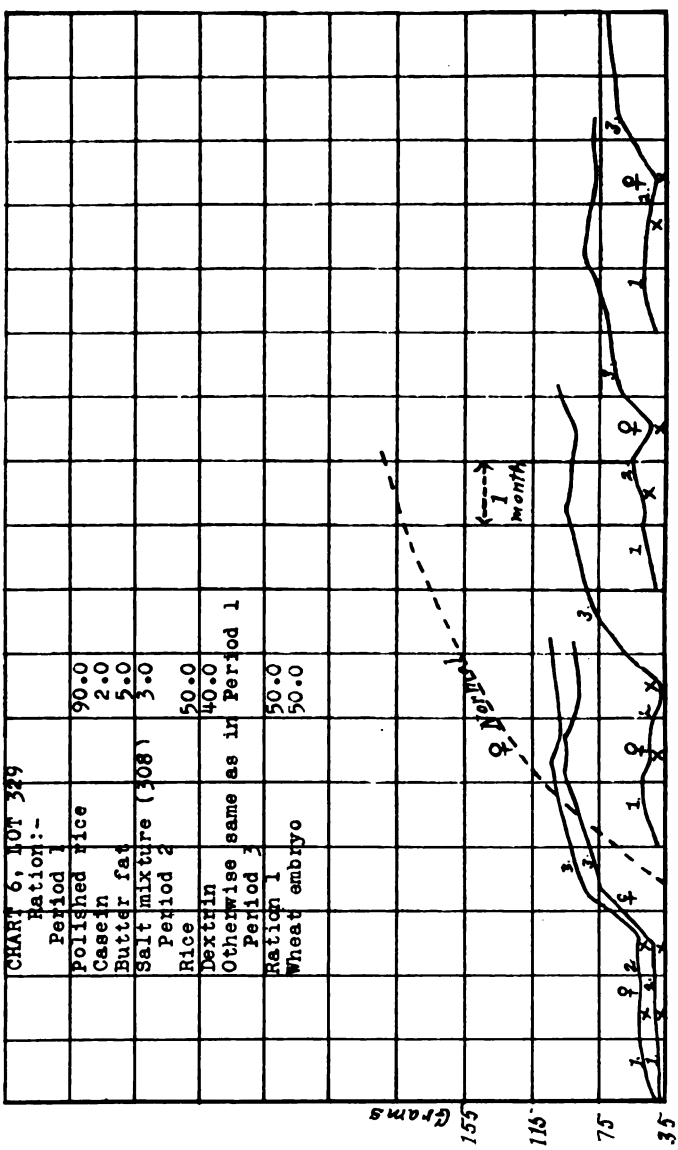


CHART 6. Lot 329. Period 1 illustrates the failure of nutrition of rats fed polished rice supplemented with purified foodstuffs. The inclusion of 2 per cent of casein in a ration closely similar to that of Lot 317 (Chart 5) does not lead to growth.

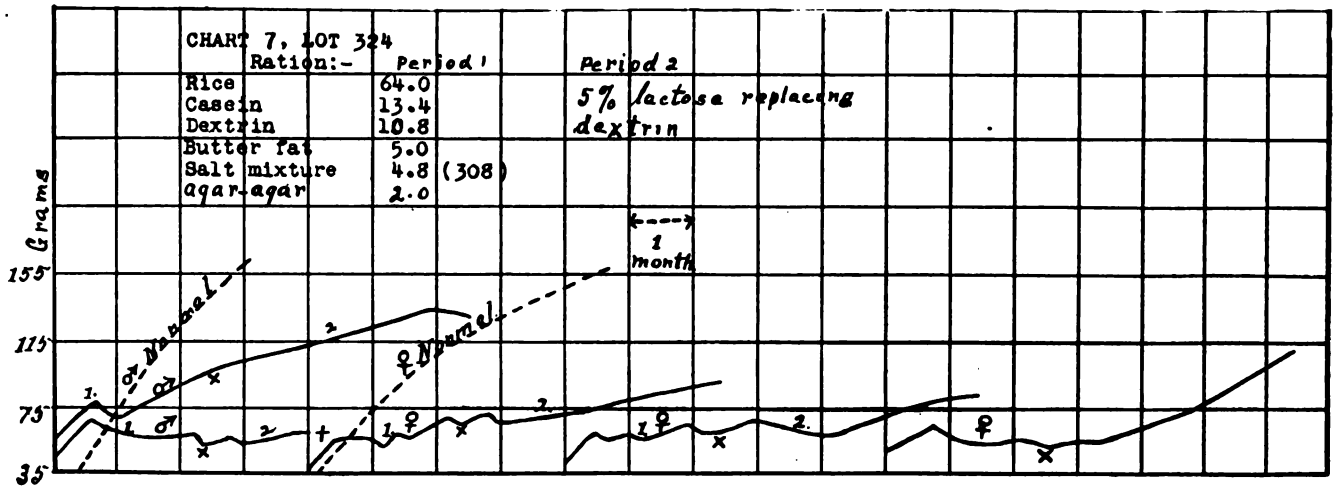


CHART 7. Lot 324. In Period 1 polished rice was supplemented with a liberal amount of purified casein, and with butter fat and suitable salt mixture additions, yet almost no growth resulted. A marked change in the rate of growth followed the introduction of 5 per cent of lactose (Merck's) into the ration in Period 2. Lactose of this purity still contains a growth-promoting accessory. (Compare with Lot 340, Chart 8.)

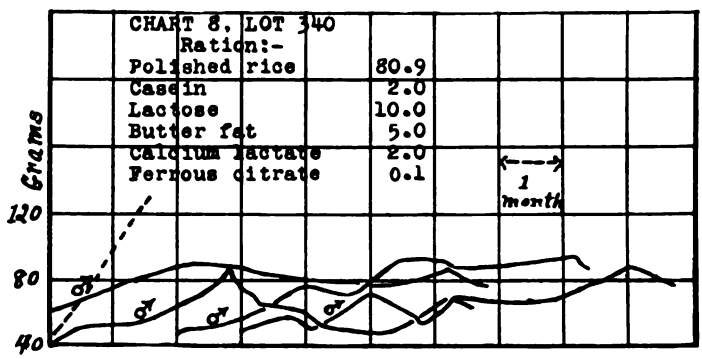


CHART 8. Lot 340. This ration differed essentially from that of Lot 329, Chart 6, only in containing 10 per cent of lactose replacing its equivalent of polished rice. The improvement in well-being and the slow growth during two or three months show that lactose of the purity of ordinary reagents (this lactose contained about 0.034 per cent N) still carried some substance having pronounced biological value in promoting growth and sustaining well-being.

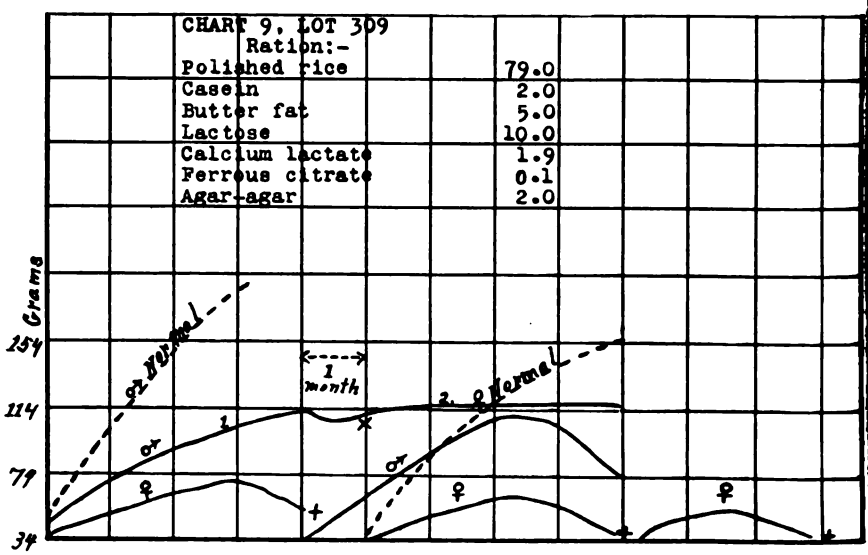


CHART 9. Lot 309. This group of rats manifested a marked improvement over those previously described, an exceptional individual reaching nearly half the normal adult size and maintaining this weight until he was nine months old. This improvement was apparently due to the lack of purity of the lactose which the ration contained. (Compare with Lots 324 and 340, Charts 7 and 8.)

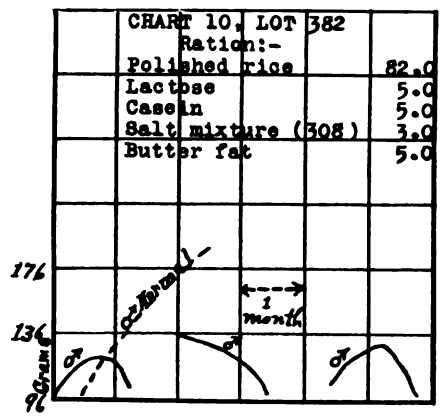


CHART 10. Lot 382. This ration is similar to Lot 329 (Chart 6) but carried more casein (5 per cent) and 5 per cent of Merck's lactose. There is no noticeable improvement as a result of these modifications of the diet. (Compare Lot 329, Chart 6.) These results indicate that lactose itself is unnecessary during growth. This is also borne out by feeding experiments with egg yolk alone on which good growth is attained. Egg yolk contains no lactose.

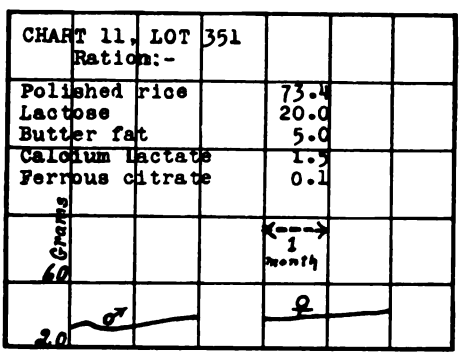


CHART 11. Lot 351. This lot which failed to increase their body weights on a ration of rice, salt mixture, butter fat, and 20 per cent of lactose, apparently did so because of the low protein content of the ration. (Compare with Lot 324, Chart 7.)

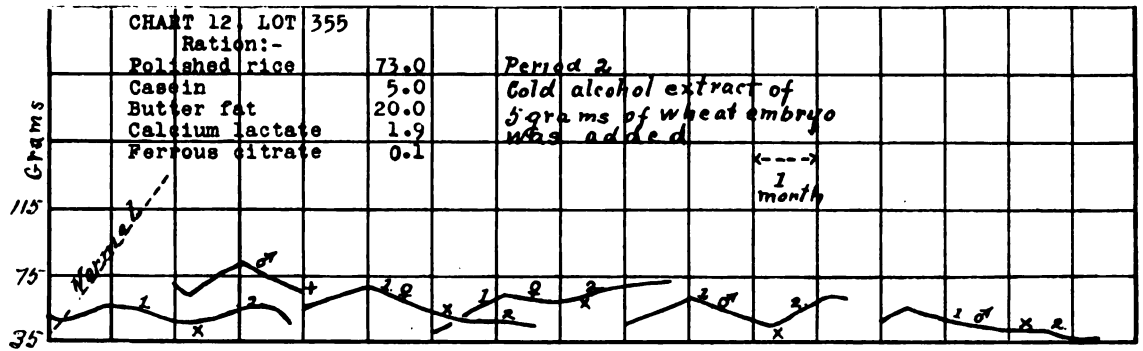


CHART 12. Lot 355. The curves here exhibited established the fact that the unknown accessory foodstuff carried by the butter fat is not the only one required by the growing animal. Increasing the butter fat content to 20 per cent in a ration made up of rice plus purified foodstuffs, does not induce growth. Another accessory which is present in small amounts in lactose of commercial purity is also required. This confirms the observation of Stepp[14] who found with mice that the addition of the ether extract alone to an ether-alcohol extracted ration did not prolong life.

[14] Stepp, *Ztschr. f. Biol.*, 1912, lvii, 151.

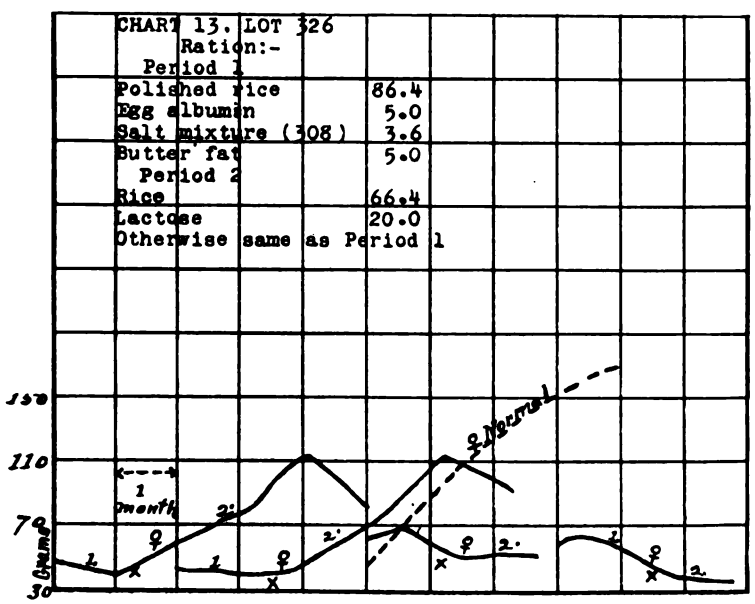


CHART 13. Lot 326. These curves support what was shown in Chart 12, Lot 355; *viz.*, that purified protein, fats, and salt mixtures cannot supplement polished rice so as to induce growth. Note the decided improvement in rate of growth in Period 2 when 20 per cent lactose was introduced into the ration. (Compare with Lots 340, Chart 8, 324, Chart 7.)

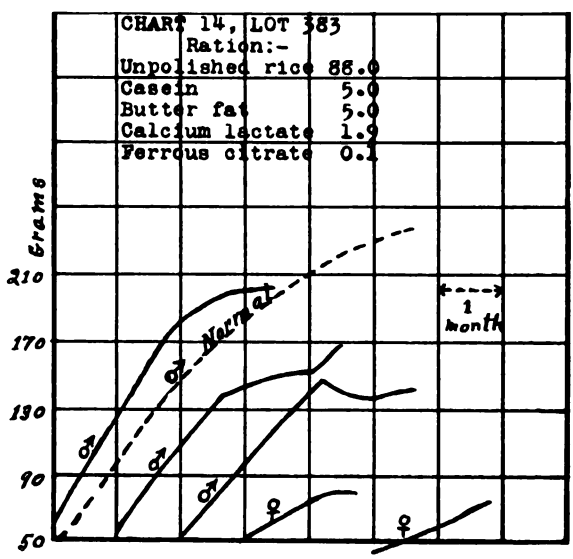


CHART 14. Lot 383. In marked contrast to Lots 326, 355, 324, and 329, Charts 13, 12, 7, and 6, all of which make it evident that purified proteins, fats, and salt mixtures cannot adequately supplement polished rice so as to induce growth, are the records here shown which reveal the fact that unpolished rice is adequately supplemented by such additions. Unpolished rice behaves in this respect like whole wheat which we have previously shown is made adequate for complete growth and normal reproduction and rearing of the young, by the addition of purified casein, a salt mixture, and butter fat.[15]

[15] McCollum and Davis, *Jour. Biol. Chem.*, 1915, xxi, 615.

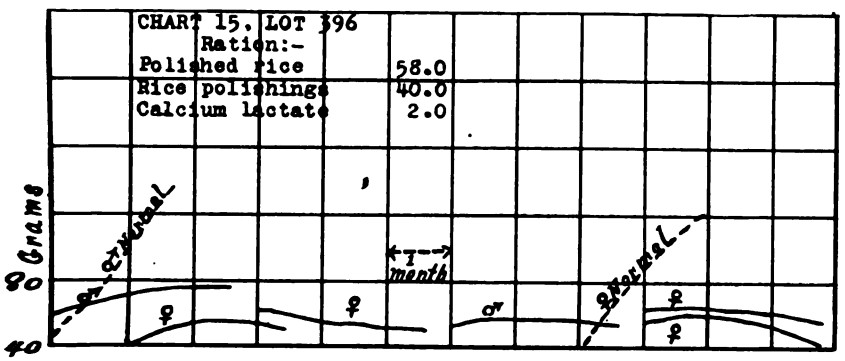


CHART 15. Lot 396. That rice polishings in amount sufficient to supply about 5 per cent of fat do not furnish the fat-soluble accessory, at least in adequate amount, is indicated by the curves of the rats of this lot. Rice polishings evidently supply the water-soluble accessory (Lot 383, Chart 14), but without the addition of the fat-soluble one no growth was attained with this ration. (Compare with Lots 392 and 395, Charts 16 and 17.)

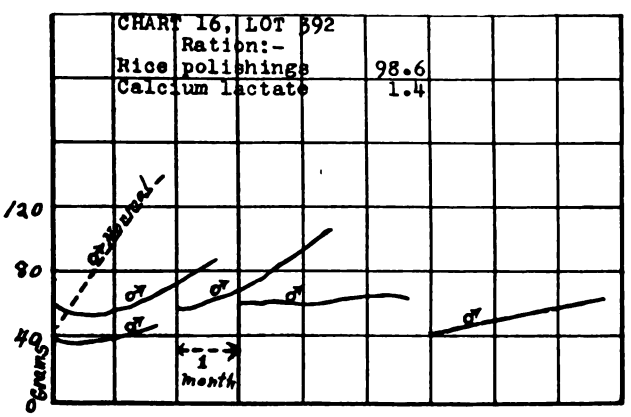


CHART 16. Lot 392. These curves indicate that rice polishings with calcium lactate are not toxic to young rats. The failure of Lot 396, Chart 14, to grow on a ration containing 40 per cent of this constituent cannot, therefore, be attributed to any injurious effect of rice polishings. (Compare Lot 396, Chart 15, and 395, Chart 17.)

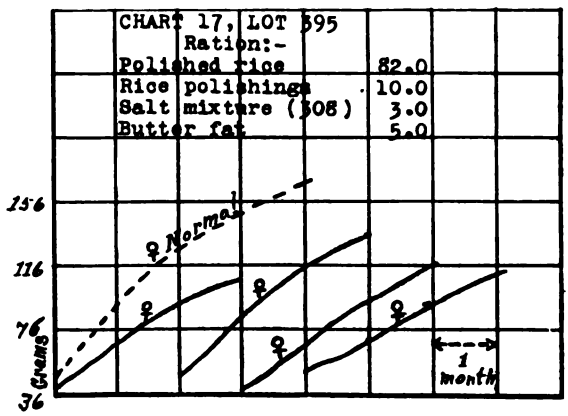


CHART 17. Lot 395. These curves demonstrate the power of rice polishings to supplement rice, butter fat, and salts, and make a ration on which good growth is attained. It seems evident that the fats of rice do not carry the fat-soluble accessory, at least in appreciable amounts. (Compare with Lot 396, Chart 15, and 392, Chart 16.)

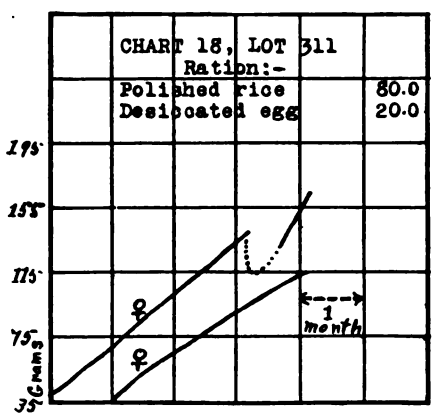


CHART 18. Lot 311. Illustrating the good growth of rats confined to a mixture of polished rice and desiccated egg. The young produced by one of these females were eaten by the mother. Our experience with reproduction on other rations has shown that rations adequate for growth are not necessarily so for reproduction and rearing of the young.[16] In discussing the curves reported in this paper we reserve all conclusions respecting the adequacy of the rations for reproduction.

[16] McCollum and Davis, *Jour. Biol. Chem.*, 1915, xxi, 615.

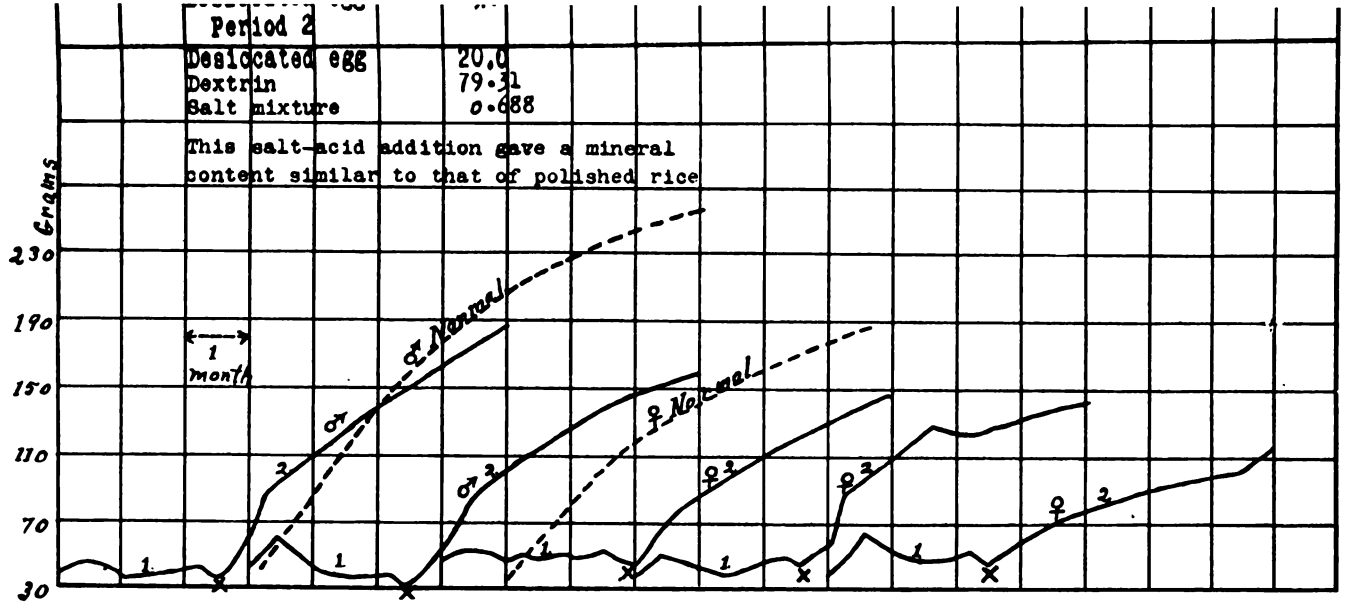


CHART 19. Lot 323. It is evident from these curves, Period 1, that 5 per cent of desiccated egg,[17] is not sufficient to supply the water- and fat-soluble accessories in the amounts necessary for growth. A comparison of these records with Lot 381, Chart 33, eliminates the protein element as the limiting factor (compare with Lot 337, Chart 20), and in Period 2 (Ration 312) of this chart the ration carried a mineral content closely similar to that of polished rice. Since good growth was observed in Period 2 it is evident that the mineral content was not the limiting factor. Salt acid mixture:

	gm.		gm.
NaCl	0.049	$CaCl_2$	0.049
$MgSO_4$ (anhydrous)	1.760	H_2SO_4	0.008
$KHSO_4$	0.200	H_3PO_4	0.206

[17] The desiccated egg employed was of high quality and was obtained from the National Bakers' Egg Co., Sioux City, Iowa.

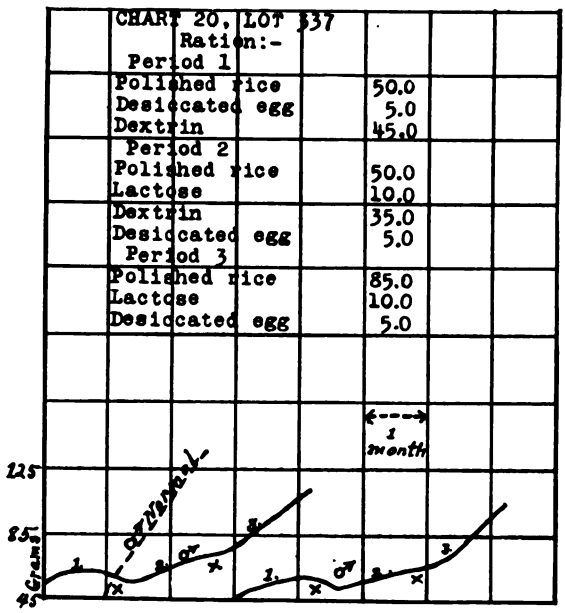


CHART 20. Lot 337. These curves illustrate in a convincing mar that lactose of fairly high purity (N content 0.034 per cent) carries water-soluble accessory essential for growth. (Compare Lots 340, Charts 8, 9.) No growth is secured in Period 1, while in Period 2 in wh 10 gm. of dextrin are replaced by 10 gm. of lactose growth at a slow rat observed. In Period 3 when the content of rice was increased to 85 per c and thereby the protein content raised, the growth rate becomes ab normal.

Lot 323 (Chart 19) shows that growth in this period is not due mer to the increased protein content, but primarily to the accessory added the desiccated egg.

In Period 2 the amount of accessories was adequate—probably near minimal limit—but the protein content limited growth. These cu show further, that 5 per cent of desiccated egg supplies enough fat-solu accessory for growth, since lactose contains none of this constituent. M than 5 per cent of desiccated egg is necessary to supply enough water-s ble accessory for growth. In regard to the relative amounts of the wa soluble accessory in milk powder and wheat embryo compare with Lots and 378, Charts 23, 24.

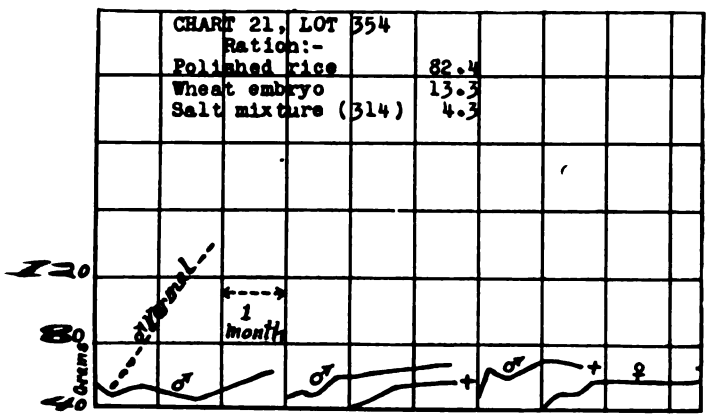


CHART 21. Lot 354. Wheat embryo to the amount of 13.3 per cent to provide an adequate amount of one of the necessary accessories growth in this ration. Reference to Charts 31, 23, Lots 339, 377, reveals fact that it is the fat-soluble accessory which is not present in this on (Lot 354) in adequate amount.

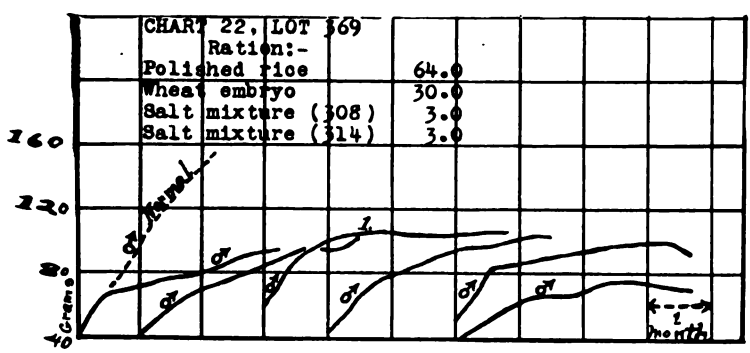


CHART 22. Lot 369. When wheat embryo is present to the extent 30 per cent in a mixture of polished rice and wheat embryo, the latter pplies enough of the fat-soluble accessory to promote growth for a time. ompare with Lot 354, Chart 21.)

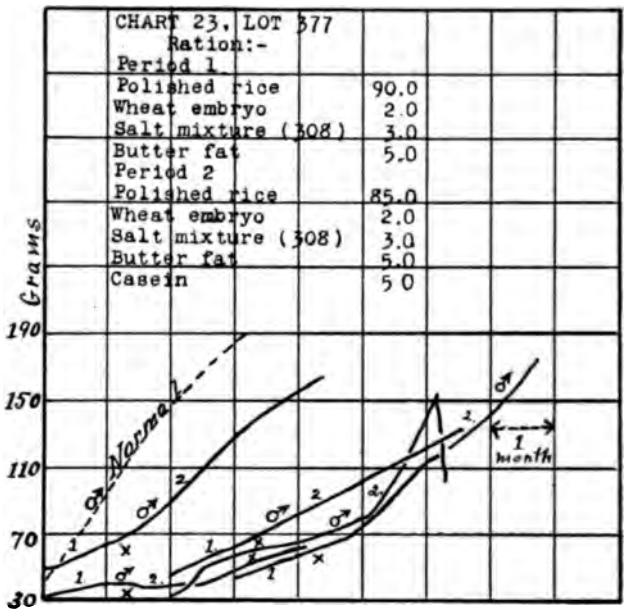


CHART 23. Lot 377. These curves show clearly that wheat embryo to the extent of only 2 per cent of the food mixture suffices to supply enough of the water-soluble accessory to enable growth to proceed at nearly the normal rate. (Compare these curves with Lot 378, Chart 24. See also Chart 9 in the following paper.) In Period 2 the rate of growth was accelerated somewhat by raising the protein content through the addition of 5 per cent of casein.

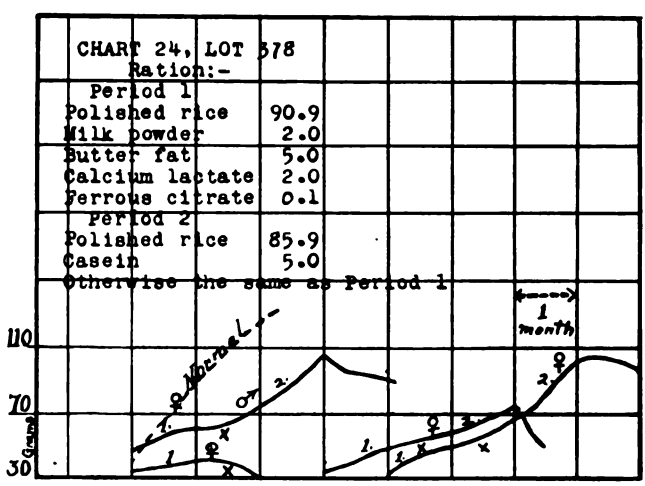
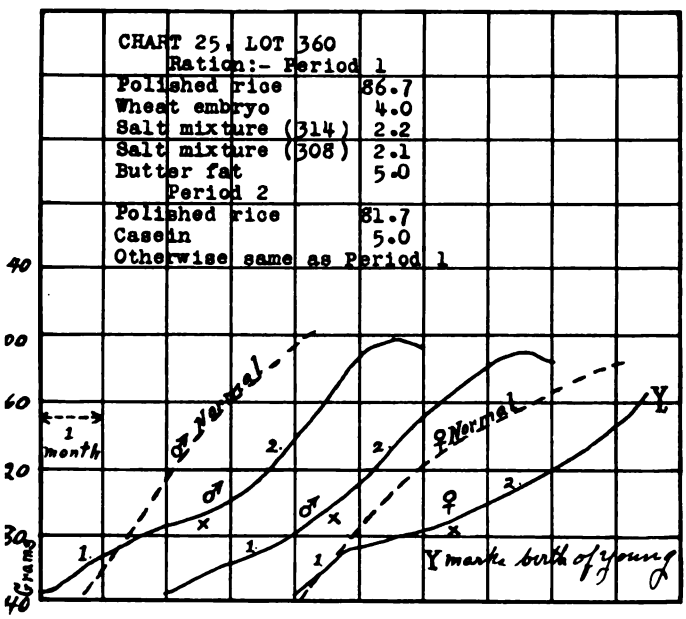


CHART 24. Lot 378. 2 per cent of skim milk powder with rice suffices to supply enough of the water-soluble accessory to induce growth nearly the normal rate.



IART 25. Lot 360. The condition with respect to growth of these which received their water-soluble accessory supply from 4 gm. of t embryo is noticeably better than those of Lot 377 which received 2 per cent of this constituent. This may reasonably be assigned to lightly higher protein content of the ration of Lot 360 derived from the ional 2 gm. of wheat embryo. (See also Lots 369, 377, Charts 22, 23.)
mixture 314:

	gm.
NaCl	1.067
K citrate	0.205
K_2HPO_4	3.016
$CaCl_2$	0.386
$CaSO_4.2H_2O$	0.381
Ca lactate	5.553
Fe citrate	0.1000

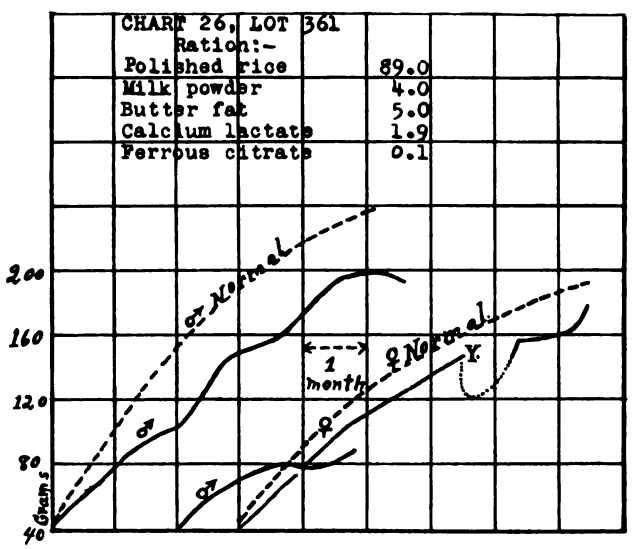


CHART 26. Lot 361. The noticeably better growth of these rats receiving 4 per cent of skim milk powder with rice, butter fat, and salts as compared with Lot 378 which received 2 per cent, may be reasonably assigned in great measure at least to the added content of protein. 2 per cent of skim milk powder contains enough of the water-soluble accessory to support growth at a fairly rapid rate.

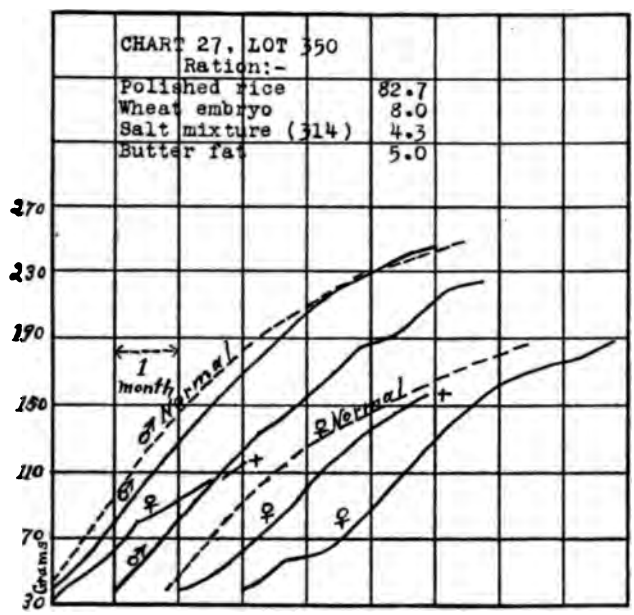


CHART 27. Lot 350. This chart illustrates how vigorous may be the growth of animals deriving their ration from polished rice, wheat embryo, butter fat, and a salt mixture. Since we have elsewhere shown[18] that the fat-soluble accessory essential for growth is present in corn and in wheat embryo, it is apparent that with suitable combinations entirely satisfactory growth is to be expected from certain rations derived from vegetable sources exclusively.

[18] McCollum and Davis, *Jour. Biol. Chem.*, 1915, xxi, 179.

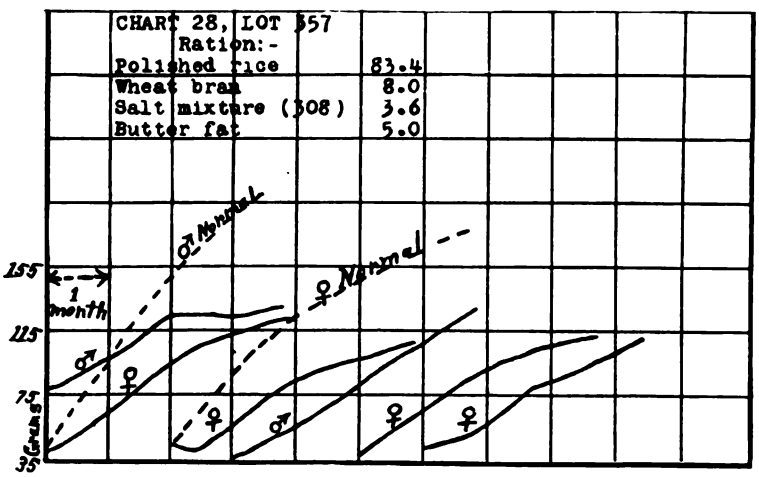


CHART 28. Lot 357. This ration was designed to show whether the water-soluble accessory so necessary to normal nutrition is present in the bran of wheat. The stimulus to growth in this lot was noticeably less than in Lot 350, Chart 27, which was given the same amount of wheat embryo as the bran content of the present ration. We have subsequently learned that in the milling process some embryo always passes into the bran. It is therefore possible that the effects here observed are in great part due to the small embryo content and not to the presence of the unknown accessory in the outer layer of the wheat kernel. We are investigating this matter further.[19]

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[19] An inspection of polished rice reveals the fact that in the process of polishing not only is the bran layer removed, but the embryo, which is easily detachable, as well. The great richness of wheat embryo in this water-soluble accessory, and its apparent absence from that portion of the wheat kernel which makes up bolted flour, exclusive feeding of which, according to Little (Little, J. M., *Jour. Am. Med. Assn.*, 1912, lviii, 2029), produces symptoms typical of beri-beri, lead us to suspect that the curative effects of rice polishings and of extracts of the same owe this property to the presence of the embryo rather than to the bran layer. This subject is receiving further attention.

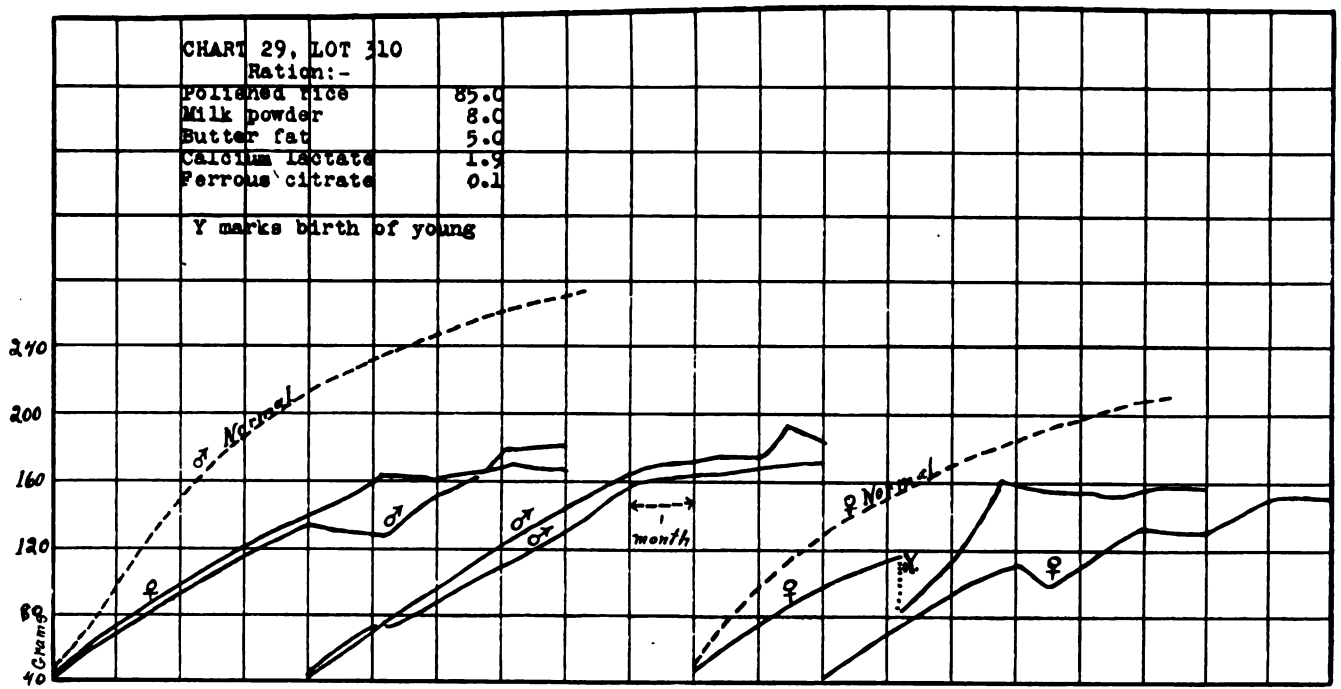

CHART 29. Lot 310. A high content of polished rice in a monotonous diet is not necessarily detrimental to a growing animal. The failure of these rats to reach normal size and support reproduction may well be ascribed to the low protein content of the ration (about 8 per cent).

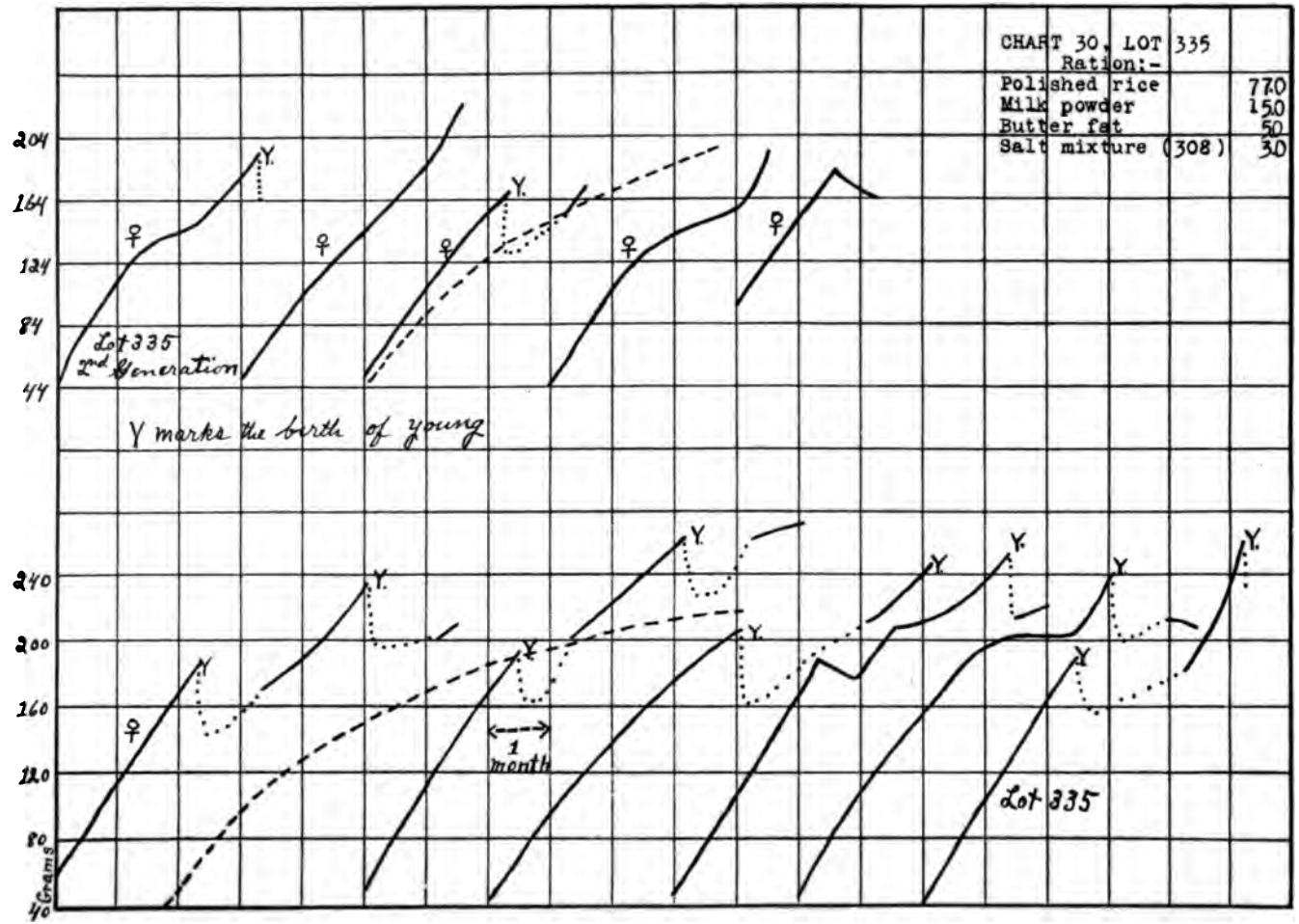
CHART 30. LOT 335
Ration:-
Polished rice 770
Milk powder 150
Butter fat 50
Salt mixture (308) 30
204
164
124
84
44
Lot 335
2nd Generation
Y marks the birth of young
240
200
160
120
80
40 Grams
1 month
Lot 335

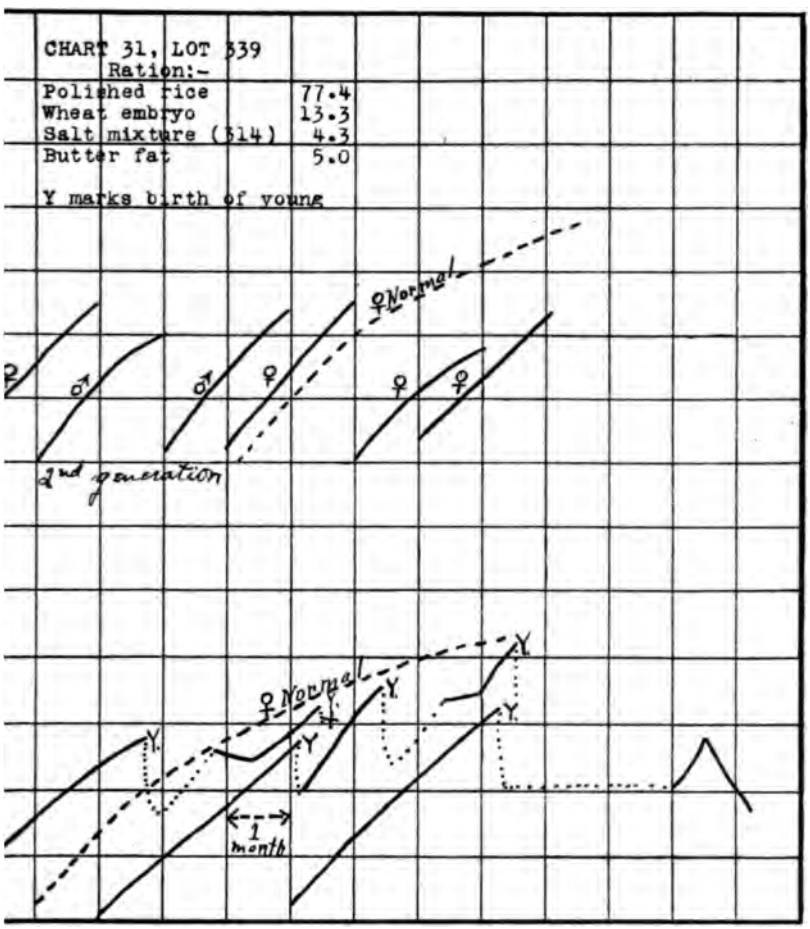

CHART 31. Lot 339. These curves show how with the addition of re fat-soluble accessory in the form of butter fat, 13.4 per cent of wheat bryo supplements polished rice in a manner so as to support nutrition sely approximating normal. When one considers that this ration conned only about 9 per cent of protein it is certainly remarkable that from ree females five litters of young were produced in the first seven months their lives. The mortality of these young was high, but one litter whose ves are shown is making good progress on the mother's diet at the pres- time.

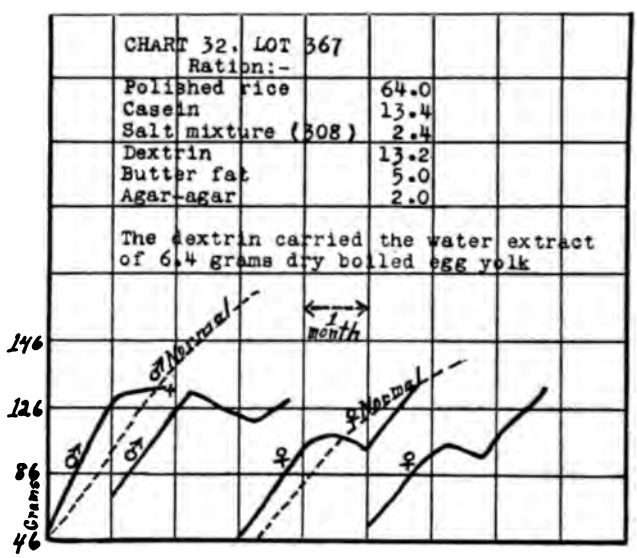

CHART 32. Lot 367. These curves show the depression in grow— due to a preparation of water extract made from slightly decomposed ▬ yolk. The recovery, as well as the initial growth, was on a preparat— from better material.

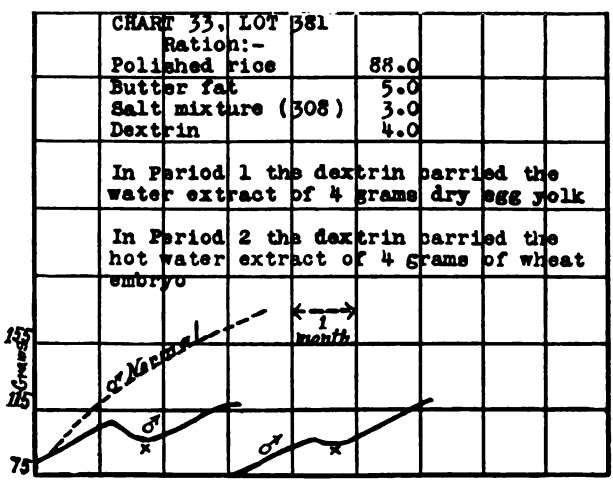

CHART 33. Lot 381. Without the addition of the accessory in the form water extract, of egg yolk, or wheat embryo, no growth is possible on this tion. (Compare with Lot 317, Chart 5.) The drop in the curves is ie result of the use of a preparation of extract made from egg yolk which ad undergone putrefaction during drying. The same depressing effect of this preparation was observed with other lots of rats on other rations. In some of these recovery and renewed growth followed changing to another preparation of extract of egg yolk prepared from eggs which were of good quality. (Compare Lot 367, Chart 32.)

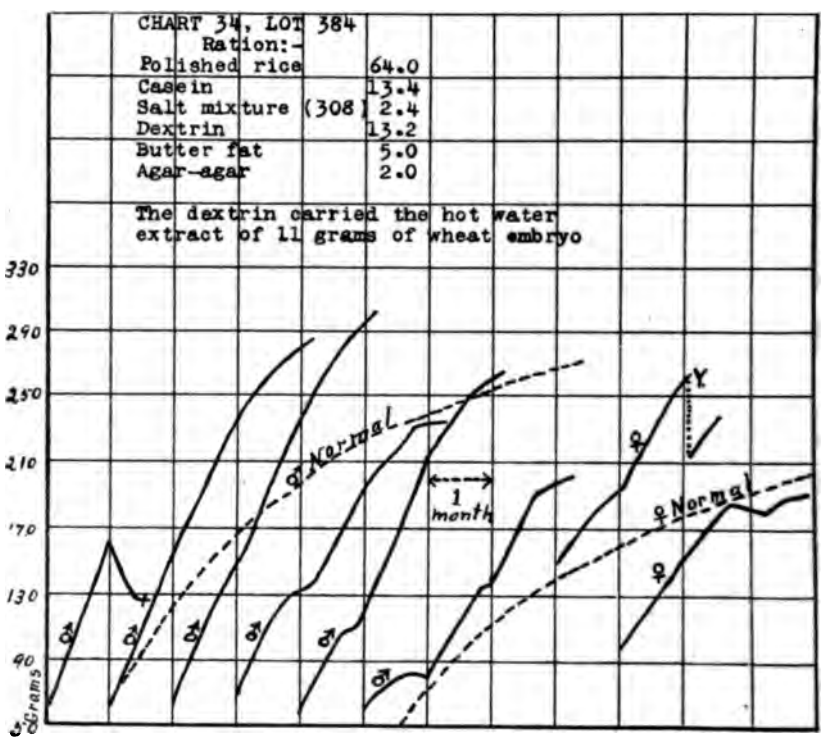

CHART 34. Lot 384. These curves illustrate the remarkable stimulating effect of water extract of wheat embryo, when added to a ration which without such addition was wholly unsatisfactory for growth. (Compare Lot 324, Chart 7.) The hot water extract was freed from protein by acidifying, boiling, and subsequent filtration.

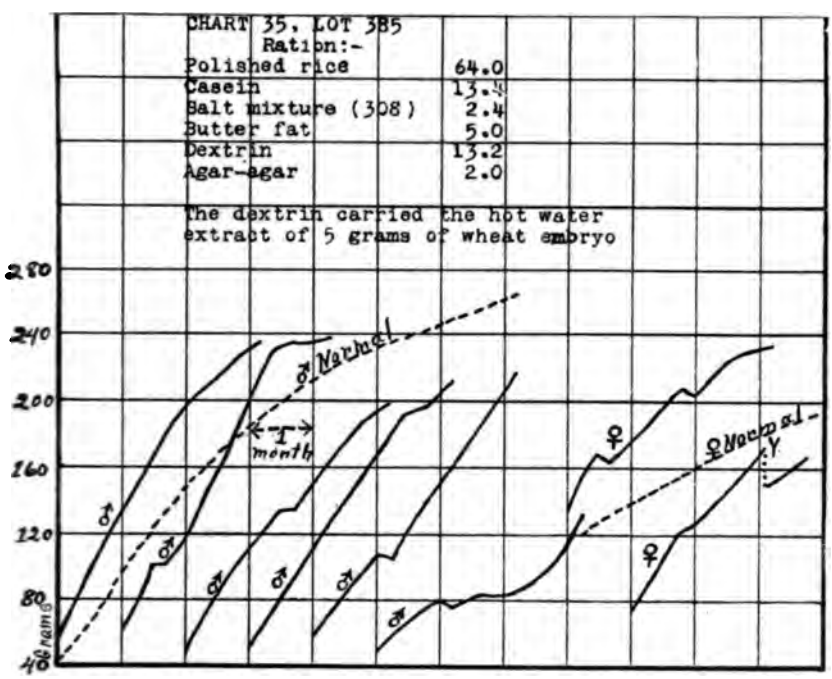

CHART 35. Lot 385. Illustrating the efficiency of the addition of the hot water extract of 5 grams of wheat embryo in promoting growth. (Compare with Charts 34 to 36.)

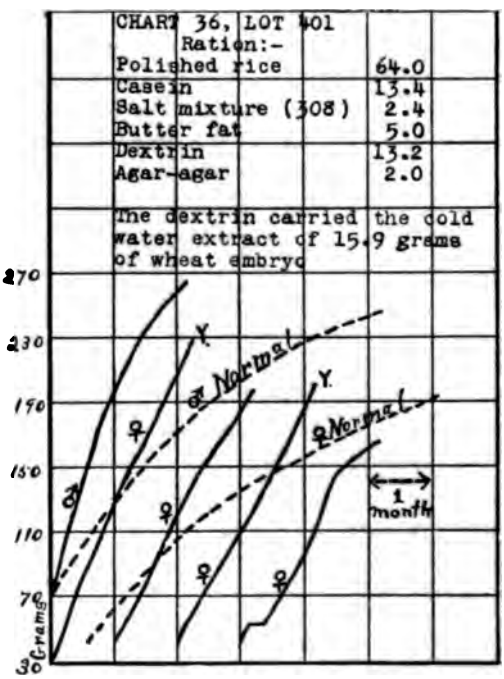

CHART 36. Lot 401. The extremely rapid growth of the rats whose curves are shown in Chart 36 indicates the ready solubility in cold water of an unknown dietary accessory present in wheat embryo. This substance is stable toward heat, for the water extracts were subsequently acidified and boiled to coagulate the proteins. To each 100 gm. of ration were added the extract of 15.9 gm. of wheat embryo.

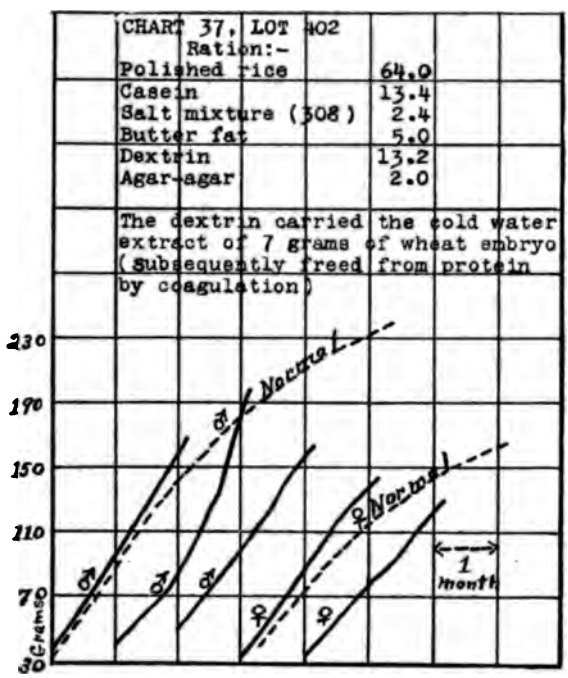

CHART 37. Lot 402. These curves show growth somewhat more rapid than the normal expectation, but not so rapid as in the rats in Lot 401, Chart 36. These rats received the same ration as Lot 401, but with the cold water extract of only 7 gm. of wheat embryo per 100 of ration.

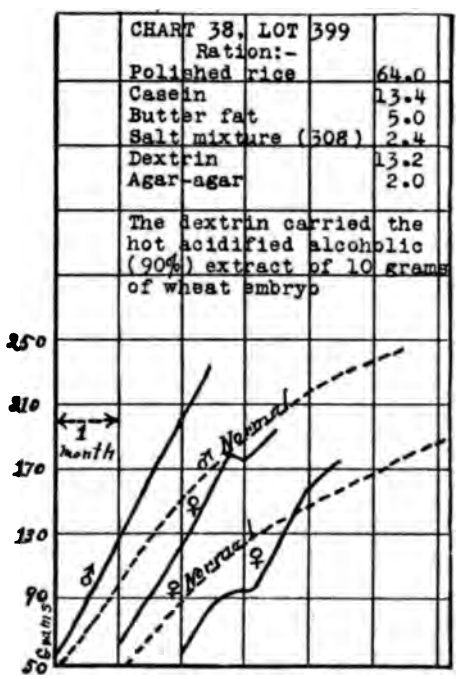

CHART 38. Lot 399. These curves illustrate in a striking manner th stimulating action on growth of a small amount of the material extracte from wheat embryo by hot acidified alcohol. This ration without th addition of an unknown accessory soluble in water and in alcohol doe not support growth. (Compare Lot 324.) The extract obtained by boiling 10 gm. of wheat embryo with hot acidified alcohol was added to each 10 gm. of ration with the result that growth proceeded much faster than th normal rate.

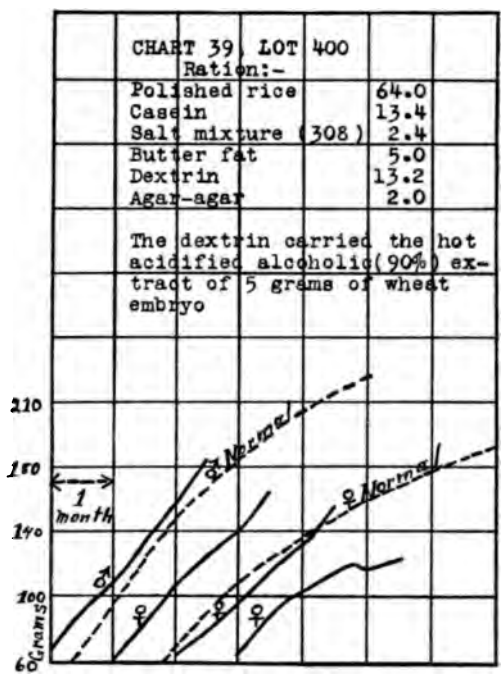

CHART 39. Lot 400. These curves should be compared with Lot 399, art 38. The rats in this lot received the hot alcoholic extract of only gm. of wheat embryo per 100 of ration, and their rate of growth was distctly slower. While very small amounts of the water- and alcohol-soluble accessory necessary for growth may suffice, it is evident from these urves that growth, at least within certain limits, is dependent on the mount present.

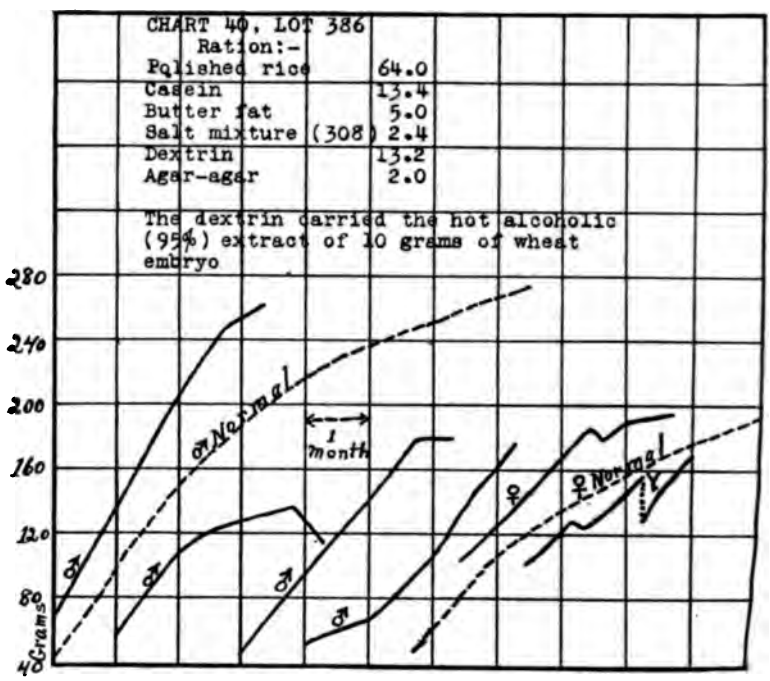

CHART 40. Lot 386. These curves should be compared with Lots 399 and 400. They received the plain alcoholic (95 per cent) extract of 10 gm. of wheat embryo per 100 of ration. The ration without the addition of the unknown accessory soluble in water and in alcohol would not have supported growth. (Compare Lot 324, Chart 7.)

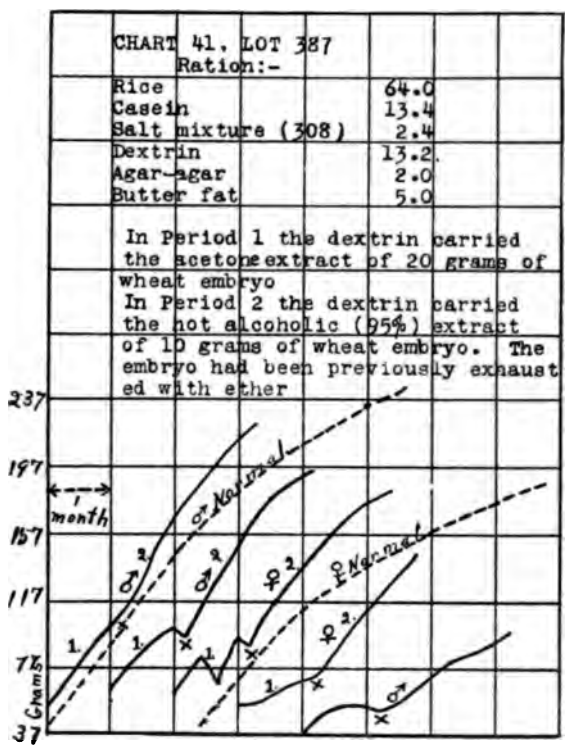

CHART 41. Lot 387. These curves show plainly that the unknown cessory essential for growth is soluble to some extent in acetone, for the dition of a hot acetone extract of 20 gm. of wheat embryo to 100 gm. a ration which would not itself support growth, induced growth at a good e during five weeks. The behavior of these animals led us to believe it they were growing on about the minimum amount possible, which umption is strengthened by the response with more rapid growth, to substitution of an alcoholic extract of half as much wheat embryo, for acetone extract. Previous to the alcoholic extraction the wheat em- o had been exhausted with ether in a continuous extraction apparatus. alcoholic extract (also by continuous extraction) was in no degree potent in promoting growth than was alcoholic extract from unextract- heat embryo. It is evident, therefore, that this accessory (water- and hol-soluble) is not soluble in ether.

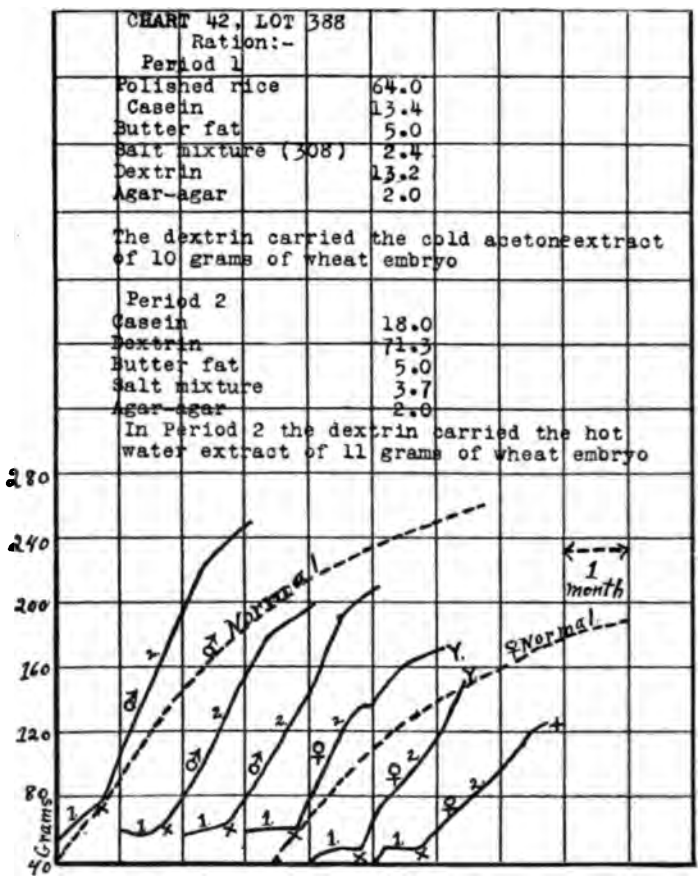

CHART 42. Lot 388. These curves should be compared with 387, Chart 41. These rats received the acetone extract of 10 gm. of wh embryo per 100 of ration (Period 1) and its influence in promoting gro was slight. In Period 2 the ration was made up of purified foodstu and was one which without the addition of this accessory, would not s port growth. The rats responded at once with excellent growth on ration when the hot water extract of 11 gm. of wheat embryo per 10 ration was added.

THE ESSENTIAL FACTORS IN THE DIET DURING GROWTH.[1]

By E. V. McCOLLUM and MARGUERITE DAVIS.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.)

(Received for publication, September 1, 1915.)

In a previous paper[2] we described experiments in which we ad attained growth approximating the normal rate, together ith reproduction and rearing of a portion of the young, with ations made up of comparatively pure proteins, dextrin, lactose, utter fat, and a salt mixture from reagent bottles. We pointed ut at that time that we suspected that the presence of lactose n our diets was the determining factor in inducing growth, although several samples showed a content of nitrogen ranging rom only 0.020 to 0.034 per cent. We stated that we reserved ur conclusion concerning the necessity of accessories other than hose carried by butter fat and certain other fats, until we should btain further evidence.

In a preceding paper[3] we have shown clearly that lactose of he purity of the ordinary reagent (Kahlbaum's and Merck's preparations) does in fact contain enough of the unknown water-oluble accessory essential for growth or prolonged maintenance, o promote growth at a fairly rapid rate when included in a diet f polished rice supplemented with casein, butter fat, and salts. Without the addition of lactose this ration does not support rowth. In conformity with this observation we found that the ation previously employed in our "nutrition with purified food-tuffs," *viz.*, casein 18 per cent, lactose 20 per cent, dextrin 56.3 er cent, butter fat 5 per cent, agar-agar 2 per cent, and salts

[1] Published with the permission of the Director of the Wisconsin Experiment Station.

[2] McCollum, E. V., and Davis, M., *Jour. Biol. Chem.*, 1915, xx, 641.

[3] McCollum and Davis, *ibid.*, 1915, xxiii, 181.

3.7 per cent, that if the 20 per cent of lactose was replaced by dextrin no growth was secured.

During the last three years Osborne and Mendel have presented numerous curves of rats fed "fat-free" diets, which indicate that the essential fat-soluble accessory is apparently stored to some extent as a reserve material, so that growth can go on for a period of sixty to one hundred days in nearly a normal manner before the supply of this substance becomes exhausted. With this view that growth can proceed for such a period with diets carrying no fats our former published data are in accord. We are now led to doubt the truth of this assertion, for reasons which will appear later.

We were convinced by our experience in feeding polished rice[4] supplemented with purified casein, lactose, butter fat, and salt additions, that all preparations of lactose were not equally efficient in promoting growth. This ration, without the lactose, promoted growth in a satisfactory manner when minute amounts of water extract of boiled egg yolk or of water extract of wheat embryo (freed from protein), or the alcoholic extract of wheat embryo were added (see Charts 32 to 42). The results all pointed to the necessity in this diet, of an accessory substance which is soluble both in water and in alcohol, as well as the necessity of the fat-soluble accessory furnished by butter fat and certain other fats.

We were convinced from these observations that the employment of lactose of relatively high purity in rations made up of foodstuffs otherwise carefully purified, is open to serious objection and that its use in such rations has led to erroneous conclusions, since it must be of exceptional purity to render it free from the water-soluble accessory.

With rations composed of polished rice, supplemented with a liberal amount of water-soluble accessory, but lacking in the fat-soluble one[5] we have not been able to secure a preliminary period of growth such as the published curves of Osborne and Mendel[6] and our own indicate to be the regular performance of rats on diets which were supposed to be free from this fat-soluble accessory.

[4] McCollum and Davis, *Jour. Biol. Chem.*, 1915, xxiii, 181.

[5] See Lot 354, Chart 21, p. 211.

[6] Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1912, xii, 8[illegible]

The "fat-free" diets of Osborne and Mendel as well as our own contained, it is true, practically no fats and seemingly insignificant amounts of lipoids of any character, but they contained either lactose or casein and in some cases both these constituents from milk and the marked difference in respect to the ability of the rats to grow during the first few weeks on these diets and their failure to do so when fed other rations known to carry but very little of the fat-soluble accessory, but carrying an abundance of the water-soluble one, strongly supports the belief that casein and milk sugar of supposed good quality still retain amounts of both classes of accessories, which are sufficient to exercise a pronounced effect on growth in young animals.

We therefore determined to examine the whole question of nutrition with highly purified foodstuffs in order to make certain whether the curves which we have presented in the past showing normal growth during periods of one to three months could be secured with rations which, in the present stage of our experience, we were convinced were entirely free from either the fat-soluble or water-soluble accessories. Our studies on polished rice had convinced us that if growth is to proceed at all both these accessories must be present.

The question as to the best method of preparing casein for such experiments arose. In the course of our work relating to the supplementary relationship between polished rice and other foodstuffs[7] we learned that prolonged heating even at temperatures of 90–100°C. could cause deterioration of the nutritive properties of milk, and by a systematic investigation we learned that the casein is the component of milk which suffers alteration during heating. For this reason we thought it unwise finally to extract our casein for a long period with boiling alcohol, as Funk and Macallum[8] have done in order to remove all traces of unknown accessory substances, since through this treatment the value of the casein may be decidedly reduced.

The method adopted was the following: Casein purified by twice repeated precipitation was washed, dried, and ground. It was then placed in a large jar having an outlet at the bottom which was closed with a plug of cheese-cloth loose enough to permit a slow passage of water through it. The jar was filled with

[7] McCollum and Davis, *Jour. Biol. Chem.*, xxiii, 247.

[8] Funk, C., and Macallum, A. B., *Ztschr. f. physiol. Chem.*, 1914, xcii, 17.

water acidified with acetic acid. When it had nearly all drai off the jar was again filled. The casein was frequently stir to prevent its forming a compact mass. This washing was c tinued during seven or eight days, the last twenty-four hou washing being with distilled water. The product thus obtain was dried and ground. It was very poor in ash, 10 gram samp yielding but a trace of calcium. By this treatment practica all the water-soluble constituents were dialyzed out of the granul

With casein prepared in this way combined with dextri butter fat, and salts we have been unable to obtain apprecia growth even during the first month. This is illustrated by Chart The results are strikingly different from those obtained with cas purified only by reprecipitation, together with liberal amou of lactose of fairly good quality. Such rations apparently ca adsorbed as impurities quantities of both classes of essential cessories which are easily detectable by the qualitative dem stration of growth in young animals.

We must, therefore, conclude with Stepp, Hopkins, Funk, a others[10] from the extensive data now available that certain present unidentified substances aside from protein, carbohydrat fats, and salts are indispensable for growth or prolonged ma tenance, and furthermore that there is a class of such accesso soluble in fats and another soluble in water and alcohol.

From the data available in our records it seems highly proba that, while the amount of accessory substances of either of th classes which is required to induce growth is small, the evide points to the belief that a certain quantity must be present bef any growth can take place, and that above this amount grov seems to be in some measure proportional to the amount of acc sories present.

It is obvious that in the study of the relative values of isola proteins fed with mixtures of purified food substances compara amounts of these two classes of accessories must be suppli Otherwise no safe interpretation can be put upon the results

[9] The dextrin was made from high grade corn-starch moistened wit 0.2 per cent solution of citric acid and heated four hours in an autocl at 15 pounds' pressure. It was then dried in a current of air at about 70

[10] Stepp, W., *Ztschr. f. Biol.*, 1912, lvii, 135; 1913, lxii, 405. Hopk F. G., *Jour. Physiol.*, 1912, xliv, 425. Funk, C., *Ztschr. f. physiol. Ch* 1913, lxxxviii, 352; 1914, xcii, 13.

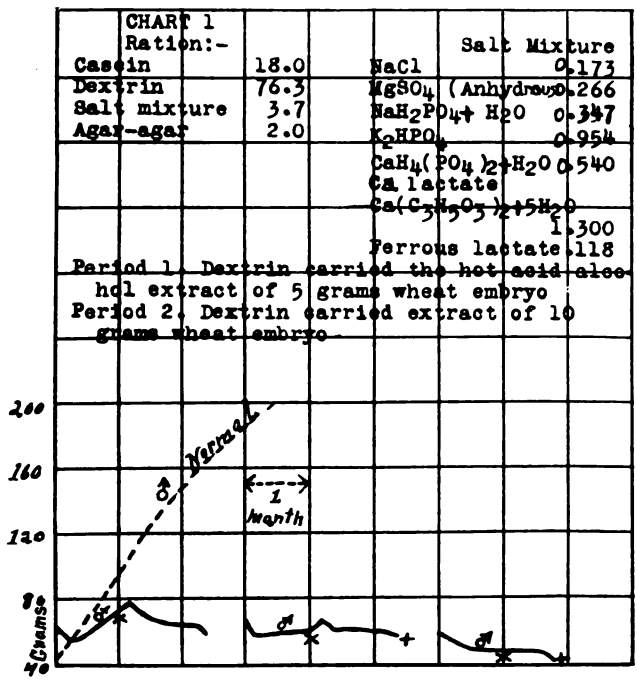

CHART 1. The curves shown in this chart are typical of the behavior of rats fed highly purified casein, dextrin, and a salt mixture. The ration was free from the fat-soluble accessory essential for growth, but a liberal amount of the water- and alcohol-soluble accessory was provided in the form of an acid alcoholic extract of wheat embryo. Growth could not proceed on this ration. Both the water-soluble and the fat-soluble accessories must be present before growth can take place. Casein and lactose of ordinary purity cannot be employed as purified foodstuffs. Growth during a period of a few weeks on diets of isolated foodstuffs is an indication that both classes of accessories are retained in the lactose and casein as impurities. A high degree of purity must be attained in order to eliminate these substances.

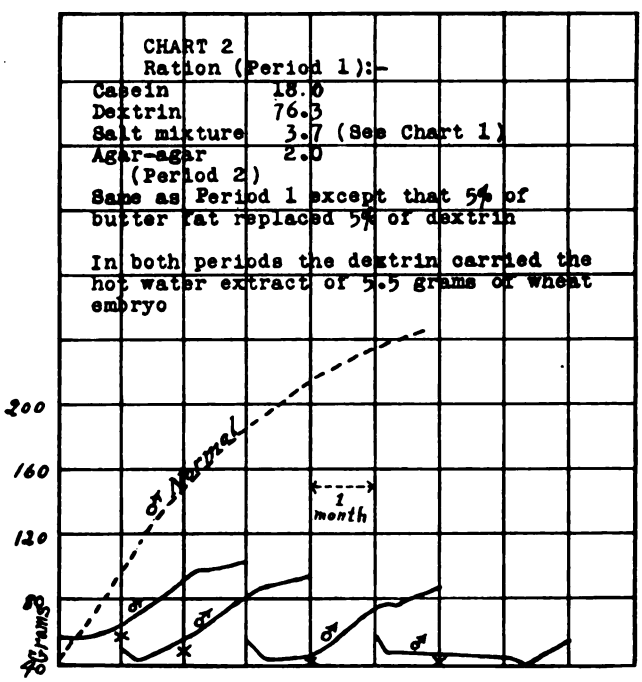

CHART 2. Illustrating the usual performance of young rats with respect to growth when fed a mixture of casein, dextrin, and salts, the casein being of exceptional purity. In Period 1 there was added sufficient water soluble accessory in the form of water extract of wheat embryo (freed from protein by coagulation) to support growth. Growth did not take place, however, because there was no fat-soluble accessory present in the diet. In Period 2 when butter fat was included in the ration growth at once began.

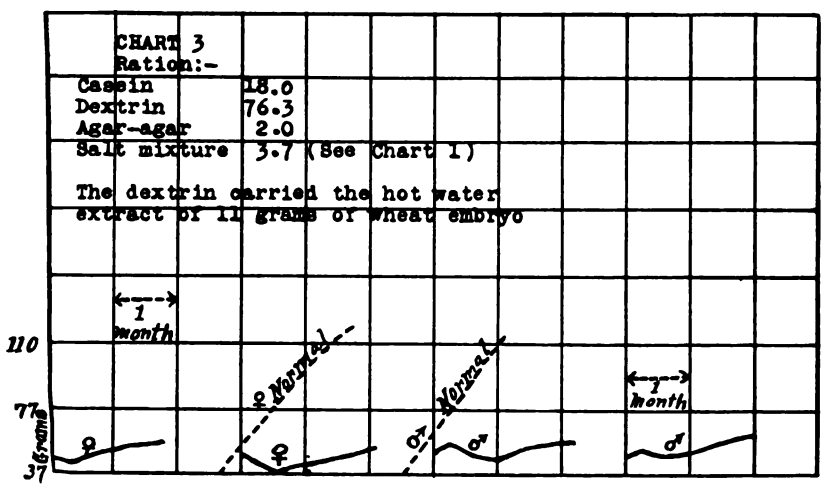

CHART 3. These curves emphasize the absolute necessity of having both classes of accessories present in the diet before growth can take place. These rats received double the amount of water-soluble accessory given to those of Lot 2, but this high intake, in the absence of the fat-soluble accessory, did not lead to growth. A comparison of Lot 355 (Chart 12, page 204) which ration contained a large amount of the fat-soluble accessory but was free from the water-soluble one shows a similar behavior.

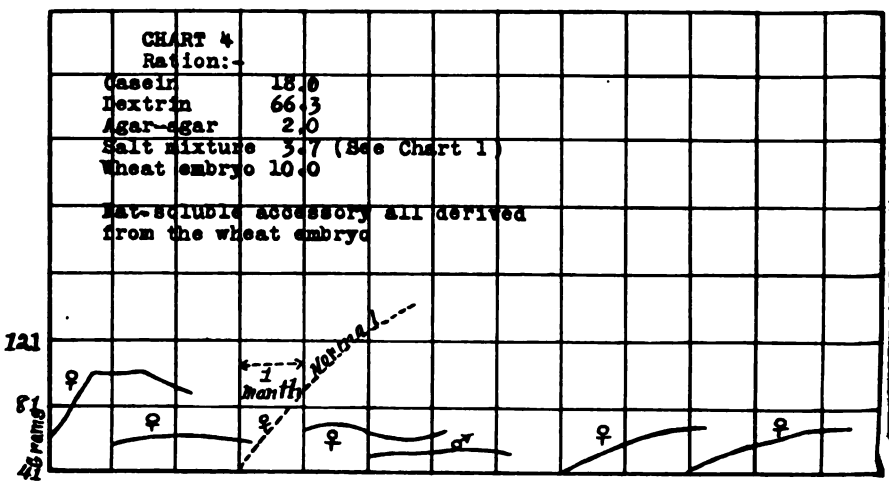

CHART 4. This ration consisting of casein (which was free from dietary accessories), dextrin, and salts, to which 10 per cent of wheat embryo was added, promotes a little growth in vigorous animals. The wheat embryo furnishes an abundance of the water-soluble accessory and a small but insufficient amount of fat-soluble accessory. Even 2 per cent of wheat embryo supplies the water-soluble accessory in amount sufficient for growth. Yet without a higher content of the fat-soluble one very little growth can be made. This ration further confirms our view that in our earlier curves and in those of Osborne and Mendel with similar rations where pronounced growth during two months or more was observed on diets containing lactose and casein, the growth was due to the fact that these components of the ration still carried small amounts of the essential growth accessories.

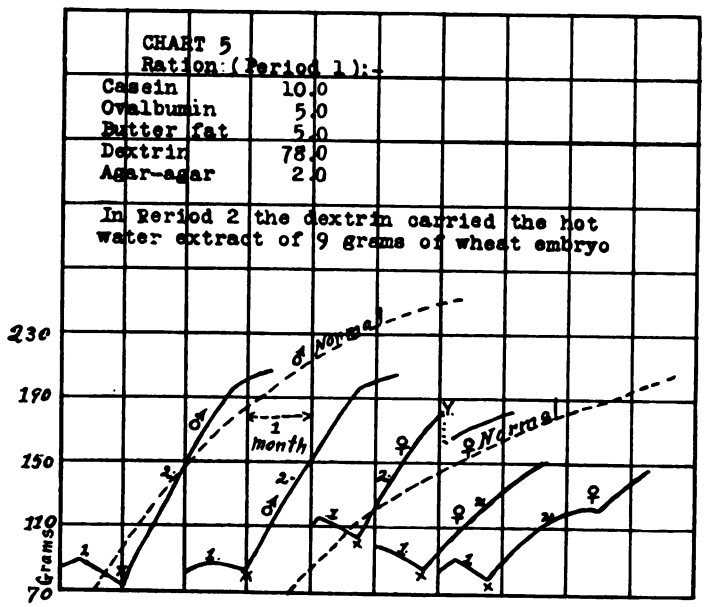

CHART 5. This ration carried adequate fat-soluble accessory with its ontent of butter fat, but in Period 1 was free from water-soluble accessory. o growth could take place. In Period 2 water-soluble accessory was ewise supplied in the form of water extract of wheat embryo (freed from otein by coagulation with heat). Growth proceeded at once at a rapid te. The evidence all points to the necessity of both classes of accessories the diet if appreciable growth is to ensue.

In this ration the nitrogen added in the form of the hot water extract of eat embryo amounted to 0.0657 gm. per 100 gm. of ration (= 2.31 per cent the total N in the ration). This is only about one-third as much nitro- n of unknown form as is added by Osborne and Mendel to their rations, herwise consisting of purified foodstuffs, in the 28.3 per cent of protein- e milk containing 0.76 per cent of nitrogen.

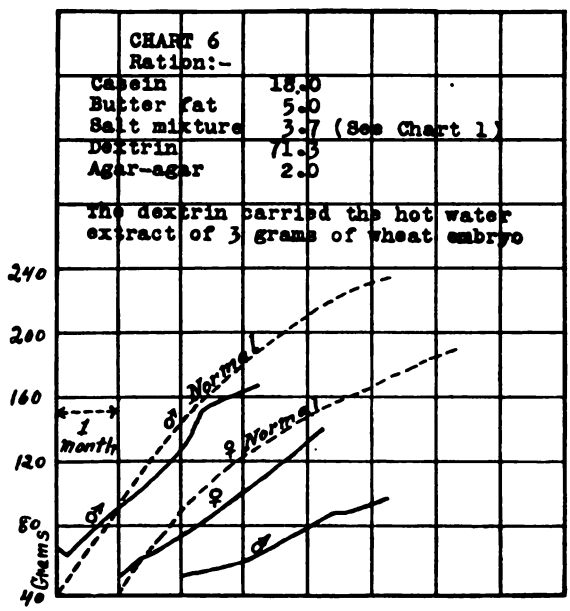

CHART 6. Shows a fair rate of growth during three months on in which all the water-soluble accessory was derived from the h extract of 3 gm. of wheat embryo per 100 of ration. 0.77 per c total nitrogen of the ration was in the unknown forms present in embryo extract. This appears to supply the accessory in amc what below the optimum.

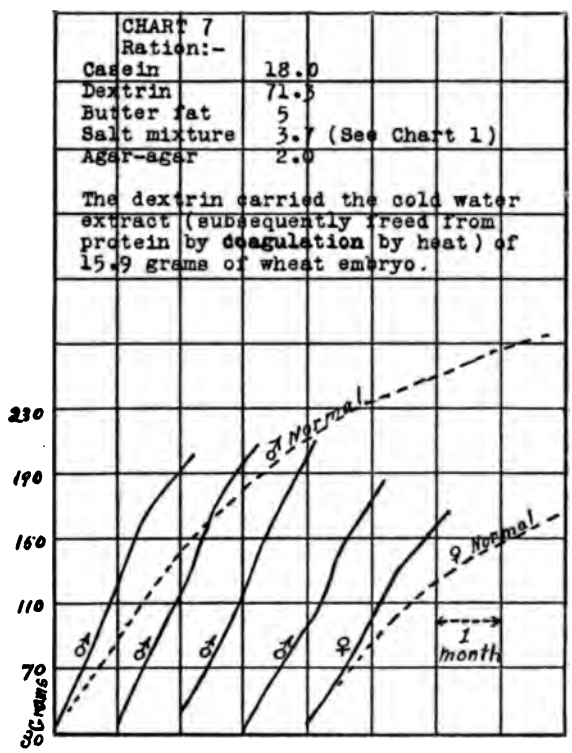

CHART 7. In the ration of these rats all the water-soluble accessory s furnished by the cold water extract of 15.9 gm. of wheat embryo per 100 . of ration, the fat-soluble one as butter fat. (The water extract was sequently acidified and boiled to remove the protein.) 4.05 per cent he total nitrogen of the ration was furnished by the embryo extract. remely rapid growth resulted from this addition, while without it no th would have taken place. (See Chart 5, Period 1.) These rats ear to be in perfect nutritive condition.

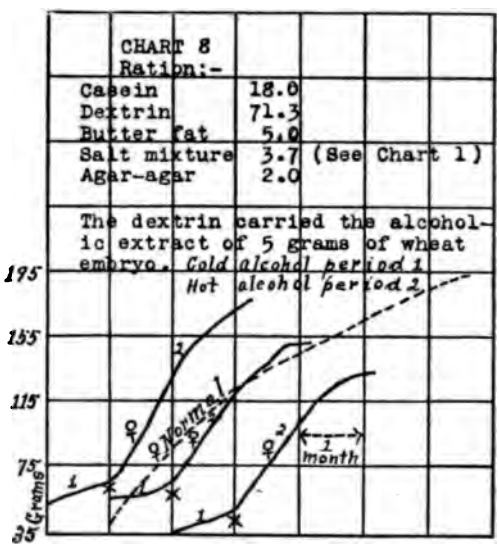

CHART 8. These curves show slight growth on a diet in which th water-soluble accessory was furnished by the cold alcoholic (95 per cen extract of 5 gm. of wheat embryo per 100 gm. of ration (Period 1) and by hot alcoholic extract of the same quantity in Period 2. This amount of ac cessory is adequate for vigorous growth. In this ration the hot alcoholi extract of wheat embryo supplied but 0.0095 gm. of nitrogen per 100 gm of ration = 0.33 per cent of the entire nitrogen content of the diet.

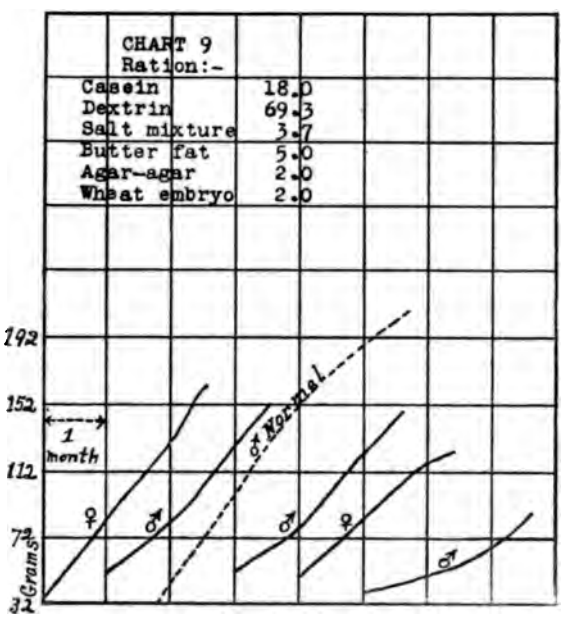

CHART 9. Showing that 2 gm. of wheat embryo per 100 gm. of ration furnish sufficient water-soluble accessory to induce vigorous growth with a diet which is otherwise satisfactory. Wheat embryo contains relatively much of this accessory and relatively little of the fat-soluble one. Similar results have been described by us in nutrition experiments with polished rice which lacks both accessories. The wheat embryo employed contained 5.1 per cent N. The nitrogen from this source was accordingly 3.57 per cent of the total in the diet.

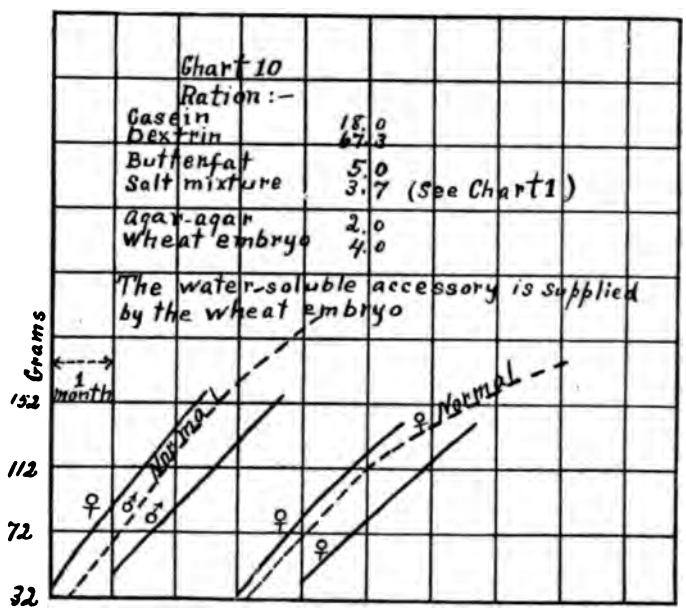

CHART 10. The ration of the rats whose curves are here shown was like that of Chart 8, except that it contained 4 per cent of wheat embryo. It did not produce growth at any more rapid rate than did the preceding one which contained but 2 per cent. It seems evident that 2 per cent of wheat embryo must supply enough of the water-soluble accessory to support growth at the normal rate.

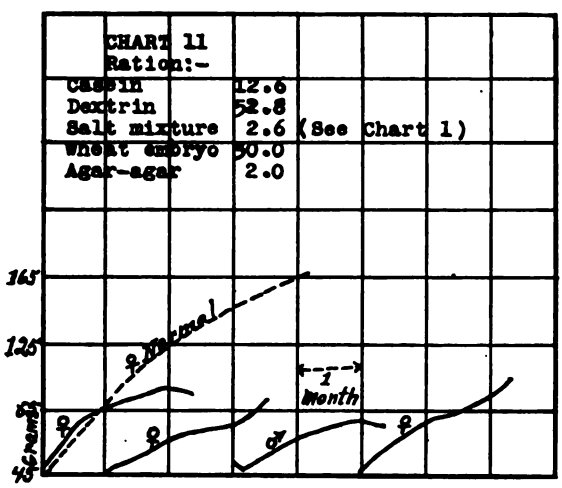

CHART 11. This chart shows that even 30 per cent of wheat embryo carrying fat equivalent to 3 per cent of the food mixture, although it supplies a certain amount, does not furnish enough of the fat-soluble accessory to enable growth to take place at the normal rate. (Compare with Chart 4.) That the rate of growth is within certain limits determined by the amounts of the accessories present is strongly supported by data which we have presented in the preceding paper (page 227).

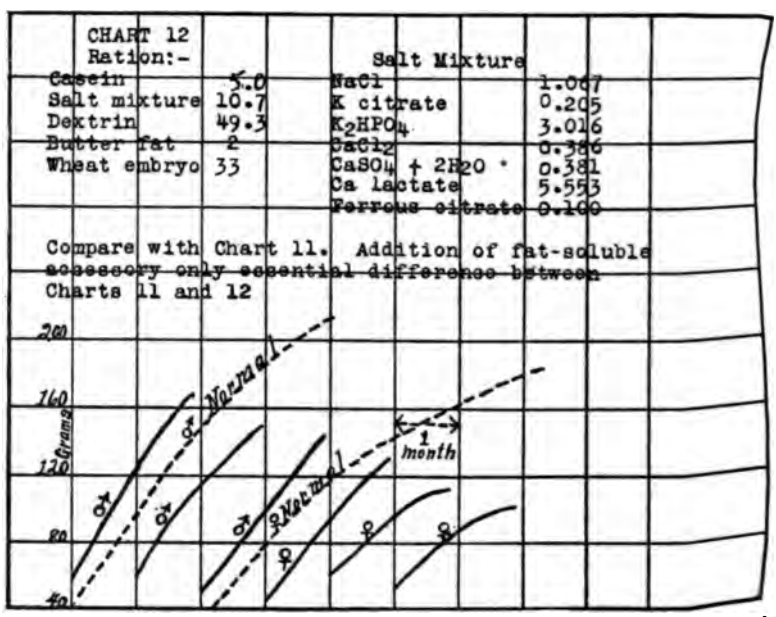

CHART 12. Lot 405. These rats received a ration entirely comparable to those of Chart 11, but the ration carried 2 per cent of butter fat. The excellent growth of these rats as compared with those in Chart 11 whose fat-soluble accessory was derived from 30 per cent of the wheat embryo, proves that it is in this particular respect that the ration of Chart 11 was deficient.

CE CAUSE OF THE LOSS OF NUTRITIVE EFFICIENCY OF HEATED MILK.[1]

By E. V. McCOLLUM and MARGUERITE DAVIS.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.)

(Received for publication, September 1, 1915.)

It has frequently been reiterated by investigators of nutrition oblems, especially in relation to scurvy and beri-beri, that e substances which when present in the diet prevent the onset these diseases, and in the early stages induce their cure, are stroyed by heating to temperatures of 115-125°C. Stepp[2] s repeatedly mentioned this; Grijns[3] states that unpolished e loses its protective power against polyneuritis when heated a temperature of 130°C. Braddon[4] states that persons eating rboiled rice did not contract the disease provided the boiling s done before the removal of the pericarp.

Numerous observations are on record to the effect that heated lk induces scurvy in infants, fresh unheated milk acting as a ative agent. Fröhlich[5] has shown that pasteurized milk (heated 70°C.) will prevent scurvy in guinea pigs fed on oats, but t milk heated to 98°C. for ten minutes fails to do so. McCol-m[6] has shown that boiled egg yolk alone induces good growth young rats. McCollum and Davis[7] have shown that the fat-uble accessory in egg yolk fat is not destroyed by boiling,

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[1] Stepp, W., *Deutsch. med. Wchnschr.*, 1914, xl, 892.

[2] Grijns, G., *Geneesk. Tijdschr. v. Nederl. Indie.*, 1901, xli, 191.

[3] Braddon, W. L., The Cause and Prevention of Beri-beri, London, 1907.

[4] Fröhlich, T., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1912, lxxii, 155.

[5] Funk, C., *Ergebn. d. Physiol.*, 1913, xiii, 125, for extensive references the literature of this subject.

[6] McCollum, E. V., *Am. Jour. Physiol.*, 1909–10, xxv, 127.

[7] McCollum, E. V., and Davis, M., *Proc. Soc. Exper. Biol. and Med.*, 3-14, xi–xii, 101.

and Osborne and Mendel[8] found that passing live steam through butter fat for two and a half hours did not destroy the fat-soluble accessory essential for growth.

From the above partial list of references it will be seen that there is no unanimity of opinion regarding the effect of the temperature of boiling water and higher temperatures on the accessory which is soluble in water and in alcohol, which is so important in the preservation of health, and whose relation to growth we have discussed in the preceding paper. In connection with our experiments designed to show the supplementary relationship between polished rice and certain other foodstuffs we observed that milk powder in quantities as small as 2 per cent of the food mixtures consisting otherwise of polished rice, purified protein, salts, and butter fat, furnished an adequate amount of the essential water-soluble accessory to induce growth. Milk powder which had been heated four hours in a double boiler was noticeably less efficient than the unheated product in supplementing the rice ration just mentioned, while when the milk powder was heated one hour in an autoclave at 15 pounds' pressure it almost entirely lost its property of supplementing this ration so as to induce growth. We observed about the same time, however, that wheat embryo, which is just as efficient as milk powder in adding the indispensable water-soluble accessory to the ration, can be heated in the autoclave in the way which renders milk valueless when added to the rice ration mentioned above, without in any way lowering its efficiency. This observation indicates that in the destruction of the nutritive value of the milk powder by heating, some factor other than the destruction of the accessory substance operated. We thereupon carried out a series of experiments designed to show what constituent of the milk powder was changed during the heating process so as to destroy its biological value.

Chart 1 illustrates the injurious effect of heating milk powder moistened with water, in a double boiler (Lot 356) and in an autoclave at 15 pounds' pressure (Lot 344). For ready comparison the curves of rats fed unheated milk powder with this ration are included on the chart (Lot 335).

Chart 3 shows that wheat embryo can be heated in a moist con-

[8] Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1915, xx, 381.

ion in a double boiler or in an autoclave at 15 pounds' pressure
:hout in the least altering its power of inducing growth when
led to the ration of rice plus purified foodstuffs. It is evident
ıt some cause other than the effect of heat on the unknown
:essory must be sought to explain the inferiority of heated as
npared with unheated milk. In wheat embryo the dietary
:essory is stable toward heat.

:n order to learn what factor is involved in the loss of efficiency
milk during heating we made a series of feeding experiments
which a ration of polished rice, casein in varying amounts,
:ter fat, and a salt mixture, was supplemented with heated
parations from milk as follows:

.. Milk from which the casein had been removed (whey), heated in
>clave.
. Milk from which the casein and albumin had been removed, boiled
hours.
. Lactose (heated in autoclave).

Ve also heated casein in an autoclave and employed it in rations
.ch when made up with unheated casein were entirely satis-
:ory.

The rice employed in these experiments had all been heated at
pounds' pressure in an autoclave.

The rations employed were of a character such that without
led protein, the rice did not carry enough of this constituent to
port appreciable growth; and with a suitable protein addition
purified proteins) no growth could take place without the addi-
ı of the essential accessory substance which can be obtained
extraction of certain foodstuffs with water or with alcohol
ıter-soluble accessory of our preceding article). We were,
refore, in a position which enabled us to detect injury to either
accessory substance or to the proteins of the food mixture
ough the effects of heat.

Lot 365 (Chart 4). These records leave no room for doubt that
ey from which the albumin has been removed by boiling
er acidifying with acetic acid may be heated at the boiling
nperature for six hours without destroying the water-soluble
:essory which completes a ration of polished rice plus purified
dstuffs so as to permit of normal growth.

Lot 370 (Chart 4) shows that whey which has been evaporate to a small volume at or near boiling temperature and then heate in an autoclave at 15 pounds' pressure for an hour is still efficien in supplying the water-soluble accessory essential to growth This result is further confirmed by Lot 374 (Chart 4), the record of which are those of rats fed heated whey with dextrin, agar-aga butter fat, and unheated casein.

The accessory substance does not appear to have been destroye to an appreciable extent through this heat treatment of the whey

In Chart 2 the marked improvement resulting from the addition of unheated casein to the inadequate diet of heated mil supports the idea that casein is the constituent of milk which impaired by heat in these experiments (Lot 260). Further support of this idea is seen in Lots 379 and 345 (Chart 5). A ratio of polished rice 77, casein heated in the autoclave 5, unheate lactose 10, salts 3, and butter fat 5 per cent resulted in a brief gai followed by sharp decline except in one individual. The appearance of these animals was very miserable.

In Lot 345 the ration was identical with that of Lot 379 (Cha 5), except that unheated casein was employed and the lactos was subjected to the heat treatment. Here we see individua which after approximately doubling their body weight have los almost none of this gain at the end of five months. From th results of the preceding paper we know that the ration in bot lots in Chart 5 contains quantities of water-soluble accessory to small to support normal growth. From these two experiment it would seem proven that casein is the constituent of milk whic suffers alteration by heat so as to lose its nutritive value.

That casein which has been heated is not highly toxic is indicate by Chart 6 (Lot 338) where is seen the effect of adding to a growth promoting ration heated and unheated casein respectively to rat otherwise nourished by a similar mixture. The heated casei did not do any appreciable injury to these rats.

Lot 254 (Chart 2) demonstrates further that milk powder whic has been heated in the autoclave is not toxic to an appreciabl degree. These rats were fed in Period 1 a ration which induce good growth, when not heated, but is incapable of doing so aft heating.

When we superimposed upon this heated ration just sufficie

milk powder to supply the maintenance needs of the rats for protein, growth at once followed for a period of six or seven weeks. The reason for this seems to be clear: the biological value of the casein was destroyed through heating in the ration fed in Period 1, and the albumin in the milk was not sufficient in amount to maintain the animals without loss in weight. When in Period 2 we gave the maintenance needs of the rats as unheated milk proteins, this quantity of protein, supplemented by the albumin of the heated milk powder was sufficient protein to induce growth for a time.

Rettger[9] and Mörner[10] have shown that casein loses sulphur when heated. It is possible that this may be associated with the loss of efficiency of casein as a foodstuff.

SUMMARY OF CONCLUSIONS.

1. Skim milk powder which has been wet and long heated in a double boiler or heated for a period of one hour in an autoclave at 15 pounds' pressure, no longer supports growth as does the unheated product. When heated, milk powder also loses its property of supplementing certain rations made up of polished rice, plus salts and butter fat; *i.e.*, rations which require both protein and water-soluble accessory to make them support growth.

2. Wheat embryo, which is as efficient as milk powder in supplementing such rice rations, can be heated for one hour in an autoclave at 15 pounds' pressure without manifesting any deterioration in this respect as does milk.

3. Skim milk from which the casein has been removed (whey) can be heated in an autoclave at 15 pounds' pressure for one hour without noticeable loss of its nutritive properties. It still supplies the water-soluble accessory in active form.

4. Whey from which the albumin has been removed by coagulation can be kept at the boiling temperature for six hours without any appreciable loss in its activity as far as the water-soluble accessory is concerned. Also lactose which has been heated in an autoclave for one hour at 15 pounds' pressure, still behaves

[9] Rettger, L. F., *Am. Jour. Physiol.*, 1901-02, vi, 450.
[10] Mörner, K. A. H., *Ztschr. f. physiol. Chem.*, 1901-02. xxxiv, 207.

as does the unheated product in supplying to rations the soluble accessory.

5. Heating casein in a moist condition for one hour in an clave at 15 pounds' pressure destroys its biological valu complete protein.

6. Heated casein or heated milk powder are shown to hav if any toxicity. The deterioration is due to a loss of value protein fraction of the ration through changes wrought i casein.

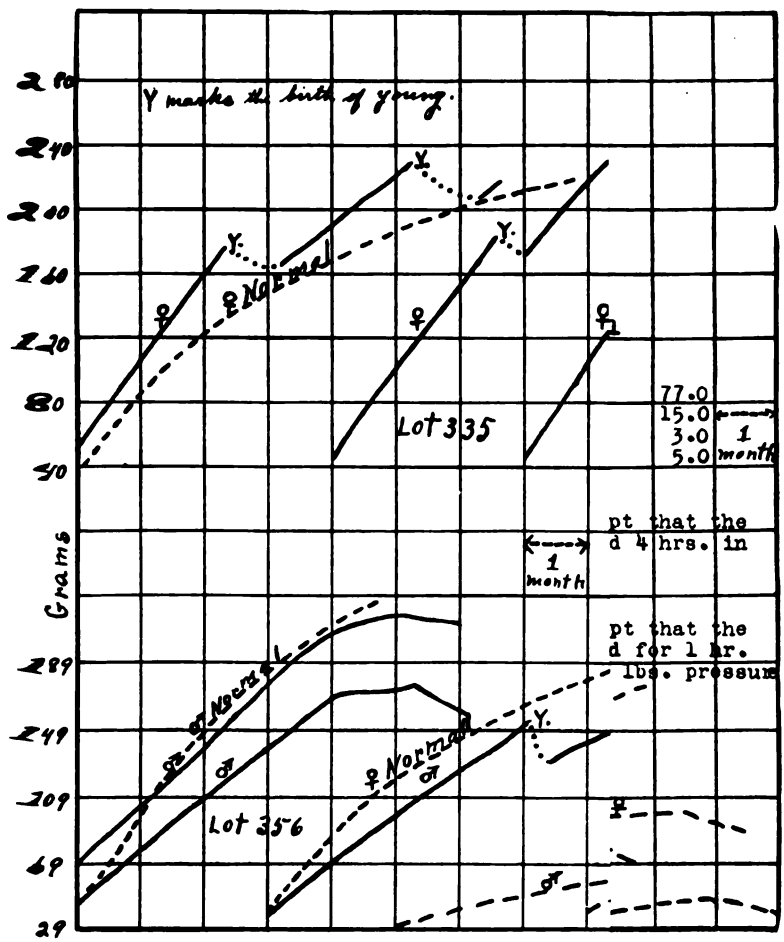


CHART 1. The curves of three groups of rats are show treatment given the milk powder in two lots.

Lot 335 illustrates the growth curves of young rats fed owder was fed as purchased, without heating. Growth was entirely satisfo litters of young; the other, one litter.

Lot 356 shows the depressing effect on growth which re a period of four hours in a double boiler, the water in which was kept bo milk thus heated, for without this constituent no growth at all would be in

Lot 344 shows the almost complete suppression of g milk powder for one hour in an autoclave at 15 pounds' pressure. The r

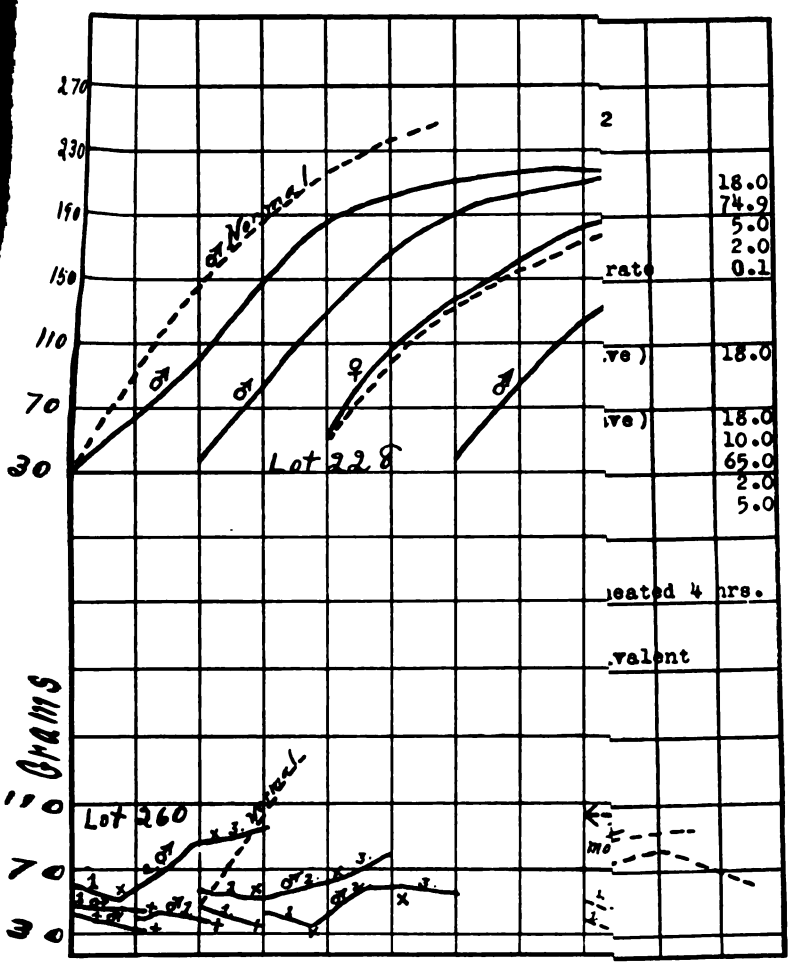
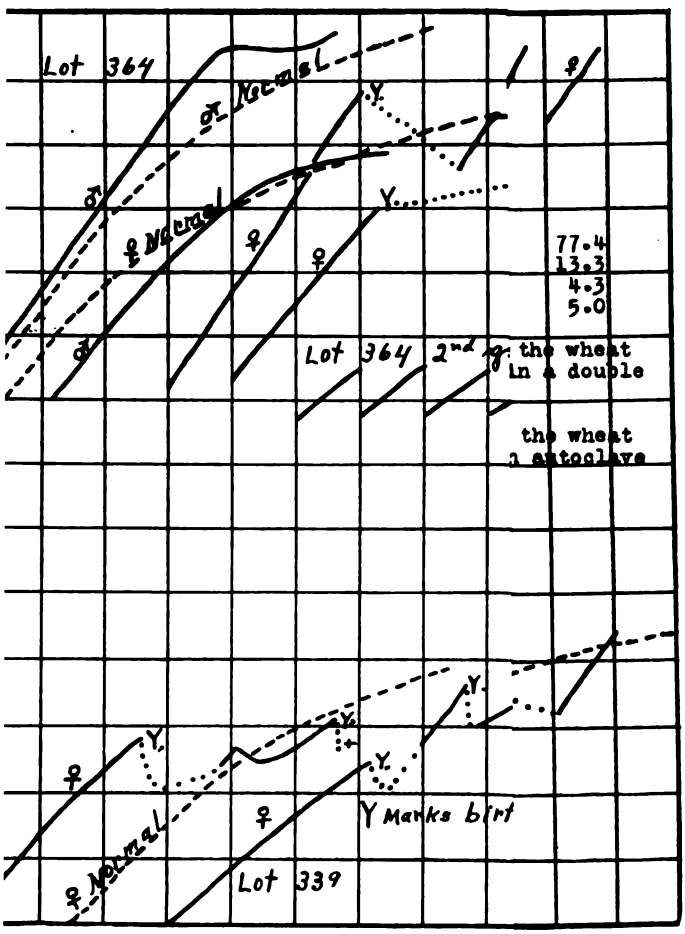


CHART 2. Lot 228 illustrates the behavior of rats ration, agar-agar, dextrin, and butter fat. All grew well during four month rats.

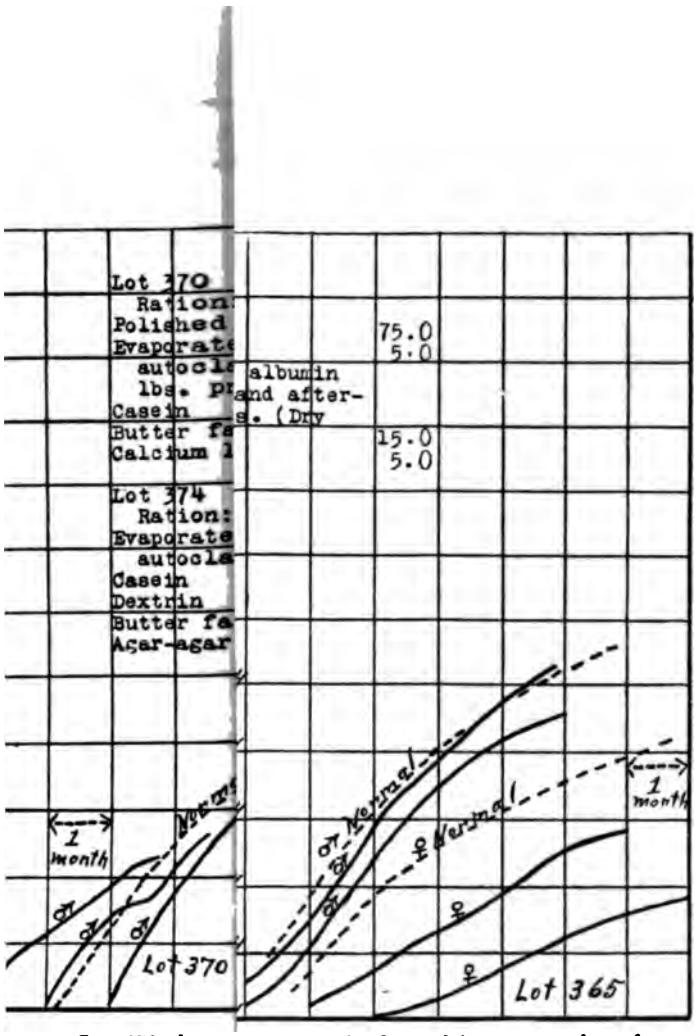
Lot 254 shows the effect on growth of heating the milk pounds' pressure (Period 1). In Period 2 the heated milk powder was sup maintain animals without loss of weight.[11] Slow growth began at once and powder is, therefore, not toxic to an appreciable degree, and furthermore, heated portion of milk powder the 10 per cent of unheated would support der supplemented with casein and butter fat induces normal growth. It, th ory and of mineral constituents to support growth. The behavior of these tment injures the protein and thereby causes a loss of its nutritive efficie

Lot 260 shows that when in a ration of milk powder and during four hours it loses its power to induce growth. The addition of 10 p wth. In Period 3 egg albumin was substituted for the casein, but without b not heated, satisfactory for growth during a long period.

[11] McCollum and Davis, *Jour. Biol. Chem.*, 1915, xx, 4



r 3. Lot 352 illustrates how wheat embryo can hring its property
menting a mixture of polished rice, salts, and buted wheat embryo,
, furnishes the water-soluble accessory in an act: powder on heat-
s property of supplementing the same rice, salts, the water-soluble
y which is essential for growth.
39 shows the records of rats grown on a ration el wheat embryo.
34 shows that heating wheat embryo in an autoclsmenting polished
ts, and butter fat so as to induce growth. Milk pie as a nutrient.
urves of six young from these rats are shown ig the first month
aning.



RT 4. Lot 370 shows whey) can be heated in an autoclave for one . 15 pounds' pressure Whey so treated when fed with polished rice heated casein, butter : food mixture without the whey would not growth or prolonged ed by the whey (water-soluble accessory) is oward heat of this in nd further supports our contention that the is the part of milk wh

374 supports the view described still serves to furnish the water- accessory and when supports fair growth during five months.

365 illustrates the grot albumin had been removed, and the remain- ition evaporated to sm Osborne and Mendel have termed "protein- lk" furnishes the wat e, pure casein, and butter fat mixture, and re supplements it so a

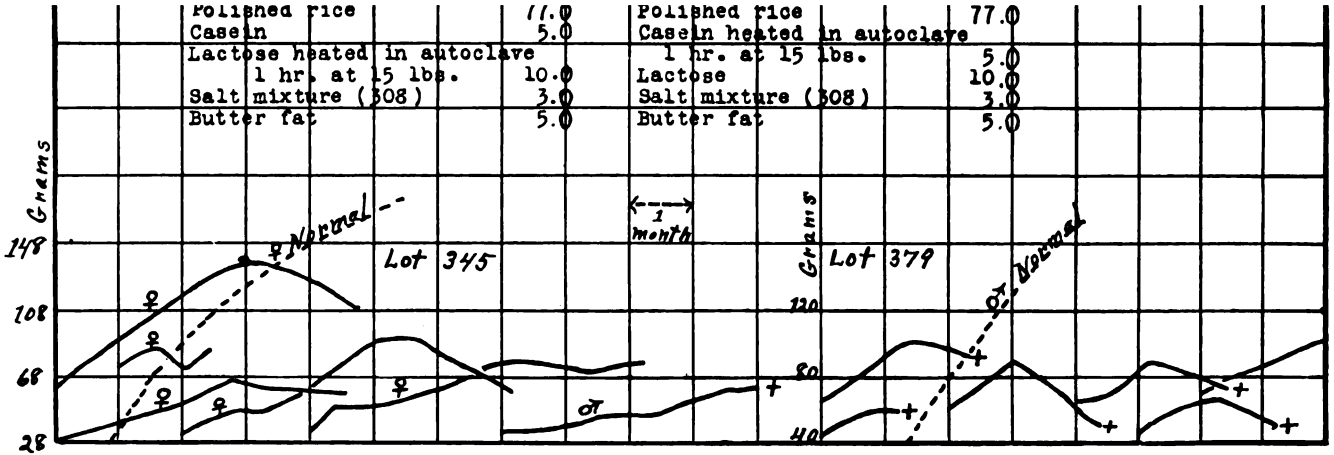


CHART 5. These curves illustrate the great difference in the nutritive properties of a ration of rice 77, casein 5, lactose 10, butter fat 5, and salt mixture 3, when the casein is heated in an autoclave as compared with the unheated. This ration carried in its lactose in the case of Lot 345 very little of the water-soluble accessory (which was not destroyed by heating). These rats made considerable growth and were able to maintain themselves for over five months. In Lot 379, in which the casein of the ration, constituting nearly half its total protein content, was changed by heat, the ration did not prevent death after about two months.

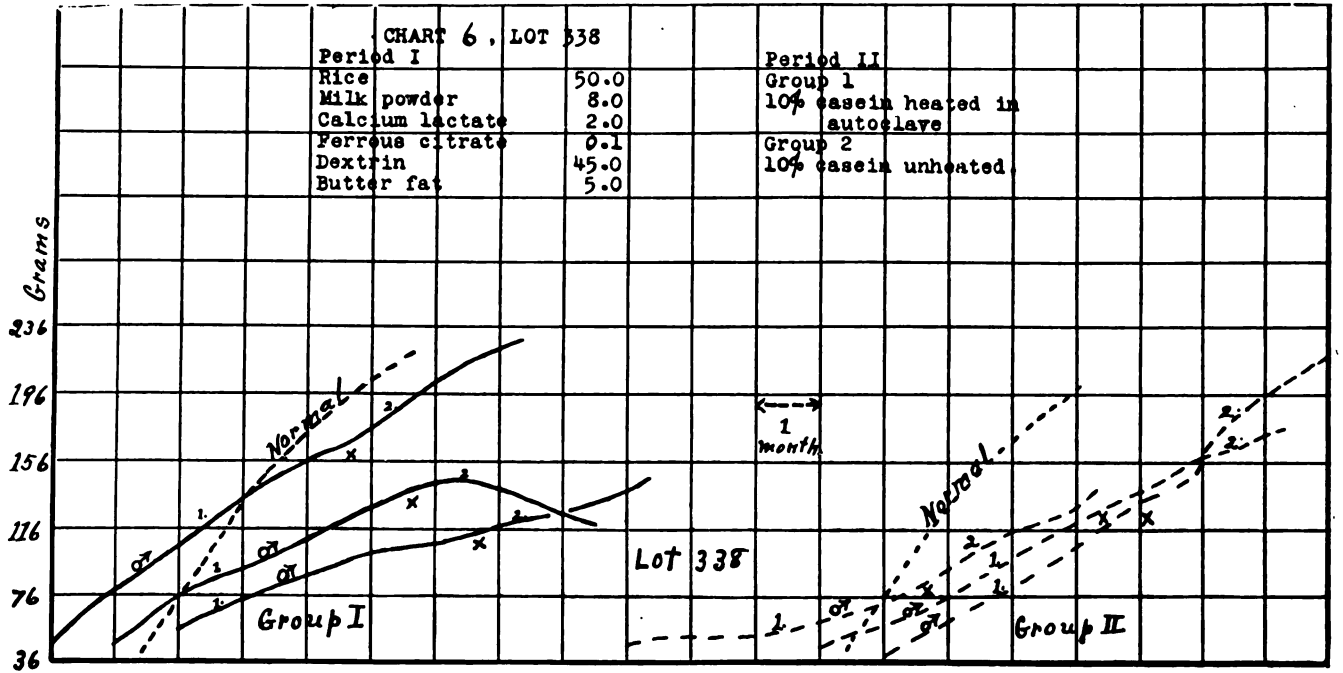


CHART 6. Casein which has been heated is not appreciably toxic to animals. This is shown by the two groups of curves shown above. Slow growth takes place on a mixture of polished rice, milk powder, dextrin, butter fat, and salts in the proportions shown on the Chart.

AN EFFECTIVE APPARATUS FOR EVAPORATING AQUEOUS EXTRACTS BY MEANS OF A CURRENT OF AIR.

PLATE 1.

BY T. B. ALDRICH.

(*From the Research Laboratory of Parke, Davis and Company, Detroit.*)

(Received for publication, August 25, 1915.)

In certain work in biological chemistry, where gland or plant tissues are to be thoroughly exhausted with water or other solvent, the writer has found it necessary to deal with comparatively large volumes of liquid, the concentration or evaporation of which for further study in the laboratory has presented some difficulties at times, owing to the sensitiveness of the active substance contained in the extract toward heat.

Vacuum concentration has its advantages where contact of the extract with air is known to be harmful, or where the body sought is volatile, and is the only method to be employed under these conditions; however where the body to be isolated or the solution to be concentrated is not exceptionally sensitive to oxidation but is sensitive to heat, the evaporation by means of a warm blast of air that may be regulated to any temperature, possesses, according to my observation, a number of advantages over the vacuum distillation method.

For example, the method is more rapid, especially at low temperatures; during the evaporation no care is necessary as in the case of vacuum distillation; the cost of evaporation is less; and over heating at any point is avoided.

Unpleasant smelling solutions may be evaporated, since the vapor is carried outside by means of a good drawing flue. The danger of contamination is slight, and may be lessened by passing the air through a suitable thickness of cotton. To avoid any danger, however, the resulting solution may be passed through

a Berkefeld filter under proper precautions in case the residu solution is to be used medicinally. In certain cases it has be customary during evaporation to add some harmless volat antiseptic, such as chloretone, which helps to keep the soluti sterile during the evaporation.

The apparatus has been employed for over a year and four very efficient in evaporating aqueous extracts of various glan

By consulting the accompanying cut and drawing to scal the construction of the apparatus will become apparent. It h in its favor especially economy of space and efficient utilizati of the heated air.

The essential parts of the apparatus consist of a motor a fan, gas burner, and two galvanized iron pipes bent in the for of a U, one enclosing the other. The smaller is attached to t fan at one end while the other end terminates in the hood and for conveying the heated air obtained from the outside roo This construction avoids mixing the air with the products of co bustion which might injure the products contained in the liqui to be evaporated. The inner pipe just below where it leaves th larger pipe divides into two pipes of equal diameter, containin gates. By this arrangement one or two blasts of air may be em ployed or one or both may be cut down as desired. The tw terminal pipes have oblong openings 8″ x 1″ respectively and bot orifices may be raised or lowered to conform to the height of th dish containing the liquid to be evaporated. All the smal pipe inside the hood may be removed in its entirety at any tim the space in the hood is required for other purposes. The ordi nary laboratory hood lends itself admirably as an exit for the ho air, since it is fairly tight and may be closed if desired, thus forc ing the heated air and moisture through the two flues (one i sufficient) to the outside air. The hood pictured has a steam bath flush with the top of the table, which may be used, if desired for heating the solution from below.

The outer pipe is covered with heavy asbestos and is soldere at one end permanently to the top of the hood; the other ope end is just above the circular burner shown in the cut.

When the burner is lit, the inner pipe is heated not only directly by the flame but also by the hot air and gaseous products of combustion which circulate freely through the outer pipe, escap-

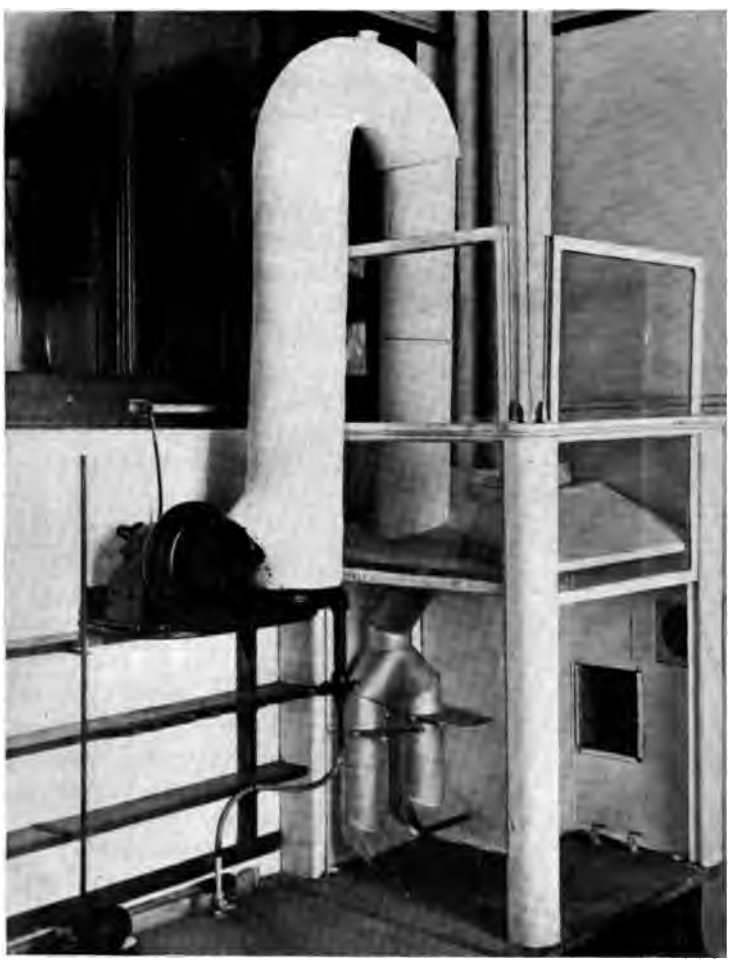


FIG. 1. Apparatus for evaporating liquids by means of a current of

(Aldrich: Evaporation of Aqueous Ext

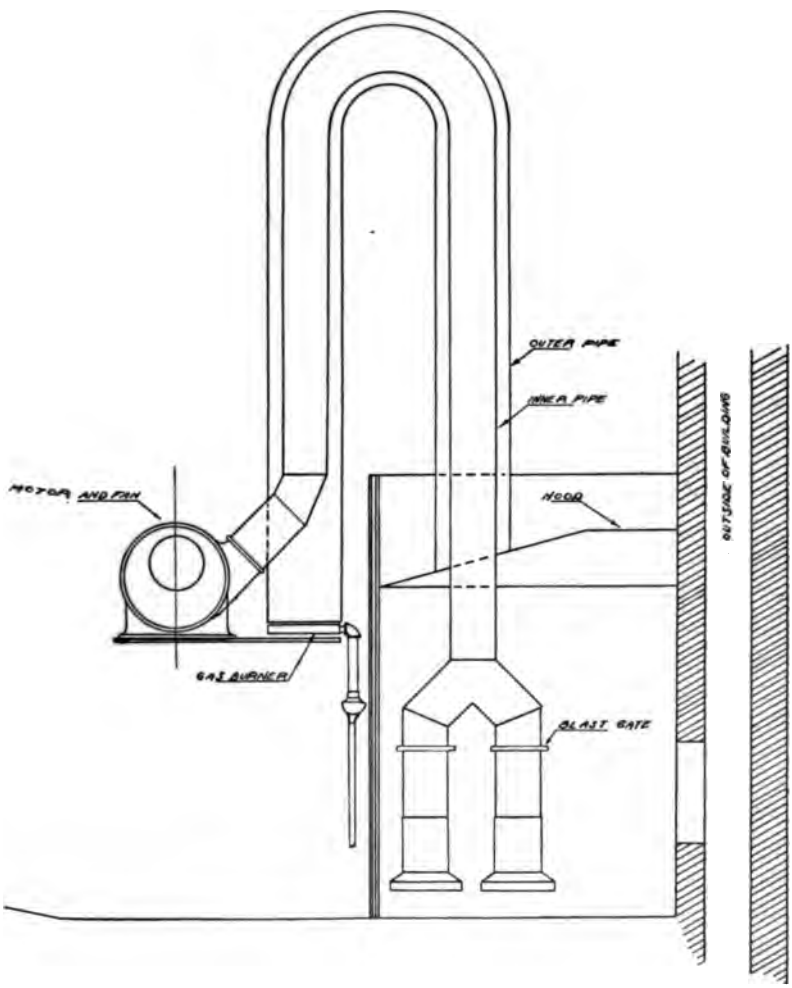


FIG. 2. Vertical section of evaporating apparatus.

ing in part into the hood and in part at the top of the oute through an opening.

The air blast is produced by a No. 1 Sirocco fan (shunt v motor) made by the American Blower Company. The m rated at 1/12 h.p. (120 watts) and produces 1,725 revolutio minute at a tension of 120 volts (1 ampere). The volu air passing through the rectangular orifices per minute is 174 feet when both gates are wide open. (Area of each opening square feet; velocity of air current 1,580 feet per minute.)

The following table gives some of the actual results ob by the use of this apparatus.

	Volume evaporated.	Time of evaporation.	No. of dishes.	Temperature of liquid.	Volum 2 d
		hrs.		°C.	
1	880	3	2	28–30	
2	560	3	1	29–30	
3	200	1	1	29–31	
4	200	1	1	31	
5	550	2	1	29–30	
6	670	2	2	26–29	
7	380	1½	2	28–30	
8	420	1½	2	28–30	

The dishes referred to are of glass and porcelain, hav bottom and the dimensions 12″ x 7½″ x 2½″. The surf of each dish is therefore only 90 square inches. From t lated results given above it is seen that even with about liquid temperature, the amount evaporated under similar conditions varies from 253 to 550 cc., or approxi 1:2. The variation can be accounted for only by the ture and the varying amount of water vapor of the air the smallest and largest volumes evaporated in one 253 cc. and 550 cc., we see that it will take from 2 to evaporate 1 liter of liquid. The eight evaporations gi age of 360 cc. per hour, equivalent to 1 liter in t If more rapid evaporation is desired, when there is r injury to the extract, a higher temperature may be heating the dishes from below, or the water surf increased.

An apparatus of somewhat similar construction was employed by Prof. Edwin S. Faust[1] to evaporate extracts susceptible to a temperature over 23°C. This apparatus (a cut of which is given on page 255 in the citation quoted) is arranged horizontally instead of vertically, and its efficiency is less than that of the apparatus employed in my laboratory. Faust states that 5 to 6 liters of aqueous extract were evaporated in 6 to 8 hours at a temperature of 22–30°. The dishes used in Faust's apparatus had a surface area of about (57″ x 12″) 684 square inches. The two dishes employed in my work have a surface area of 180 square inches or 0.26 of Faust's.

In one instance 360 cc. of aqueous solution were evaporated at 22–26° in one hour; with about four times the area this would mean 1,440 cc. per hour, providing other conditions were the same and the same efficiency prevailed. Faust's efficiency is 5 to 6 liters in 6 to 8 hours or 834–750 cc. per hour. Making liberal allowances, then, for slightly higher temperature, it would seem that the vertical apparatus is more efficient. Faust states that the cost of evaporating 6 liters of liquid is about 12½ cents or about 2 cents per liter. The cost per hour of running the motor in my laboratory is about ¼ cent and the gas consumed per hour is probably not over 1 cent, making a total of 1¼ cents for evaporating over 1 liter of water.

The efficiency of the apparatus might be further increased by placing a horizontal plate of metal as wide as the dish over the liquid to be evaporated, extending from above the rectangular opening to the further end of the dish. This arrangement should ensure closer contact of the air and water and more rapid evaporation.

My thanks are due to Mr. H. E. Whitaker of our Mechanical Department and also to Mr. C. P. Beckwith, my associate, for a number of valuable suggestions relative to the construction of the apparatus.

[1] Faust, E. S., *Arch. f. exper. Path. u. Pharmakol.*, 1904, li, 255.

XANTHOPHYLL, THE PRINCIPAL NATURAL YELLOW PIGMENT OF THE EGG YOLK, BODY FAT, AND BLOOD SERUM OF THE HEN. THE PHYSIOLOGICAL RELATION OF THE PIGMENT TO THE XANTHOPHYLL OF PLANTS.[1]

PLATE 2.

BY LEROY S. PALMER.[2]

(From the Dairy Chemistry Laboratory, University of Missouri, Columbia.)

(Received for publication, September 1, 1915.)

The pigmentation of the yolk of the egg and of the flesh (body fat) of poultry is a subject of much practical importance in the egg and poultry industry of this country. The consumer demands highly colored yolks in "fancy" eggs throughout the year, and the eggs with pale colored yolks, so frequently found on the market during the winter months, are the object of much complaint, particularly in cities. Similarly, in some sections of the country the poultry trade demands a highly colored flesh. For the fancy trade, however, the demand is for a flesh with the least color possible.

The yellow pigment characterizing the skin of hens is becoming of considerable importance in judging their egg laying activity. Thus, Woods[3] has recently stated that heavy laying invariably produces a marked reduction in the yellow color of the shank, and that, "it is possible to say positively that no bird which has been a high producer will have bright yellow legs at the end of the lay-

[1] Published by permission of the Director of the Agricultural Experiment Station.

[2] I am greatly indebted to Prof. Harry L. Kempster of the Department of Poultry Husbandry for the use of the experimental fowls and feeding equipment, and for arranging for handling the fowls throughout the entire experiment.

[3] Woods, C. D., *Maine Agricultural Experiment Station, Press Letter No. 144*, Sept. 30, 1914.

ing season." It should be pointed out, however, that Woods is mistaken in assuming that the pigment of the egg yolk and body fat of the hen is carotin, as the data presented presently will show. Similarly, Blakeslee and Warner[4] have presented data that are believed to prove conclusively that pale yellow shanks and beak, and especially ear lobes in the hen are an indication of much greater previous egg laying activity than yellow shanks, beak, and ear lobes. "The assumption is that laying removes the yellow pigment with the yolks more rapidly than it can be replaced by normal metabolism, and in consequence, the ear lobes, beak, and legs become pale by this subtraction of the pigment."

The yellow pigment of the egg yolk has recently been identified from a chemical standpoint by Willstätter and Escher.[5] These investigators isolated the principal pigment from the yolks of 6,000 hen eggs in crystalline form, and from its chemical constitution and properties believe it to be isomeric with the crystalline xanthophyll of the chloroplast. As a member of the xanthophyll group of pigments Willstätter and Escher designate the pigment "Xanthophyll B." As a distinct animal pigment, however, these authors designate the pigment as Lutein. The presence of a carotin-like pigment accompanying the Lutein in very small proportion is reported by these investigators. An excellent review of the chemical studies of the egg yolk pigment which preceded their own is given in the paper.

While the work of Willstätter and Escher probably is to be regarded as the final identification of the egg yolk pigment, the relation of the pigment to the plant xanthophylls was first shown by C. A. Schunck.[6] Some exceptionally beautiful spectroscopic studies led this investigator to classify the flower and plant xanthophylls into three groups according to the action of dilute HCl and HNO_3 upon their spectroscopic absorption bands. The three groups were designated L, B, and Y xanthophyll, respectively. Similar studies of the yellow pigment of the yolk of hen eggs, and of the blood serum of the hen led Schunck to

[4] Blakeslee, A. F., and Warner, D. E., *Science*, 1915, xli, 432.

[5] Willstätter, R., and Escher, H. H., *Ztschr. f. physiol. Chem.*, 1911-12, lxxvi, 214.

[6] Schunck, C. A., *Proc. Roy. Soc.*, 1904, lxxii, 170.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 1. Color of raw yolk on pigment-free ration at close of experiment.
FIG. 2. Color of raw yolk on carotin ration at close of experiment.
FIG. 3. Color of raw yolk on xanthophyll ration at close of experiment.

(Palmer: Xanthophyll.)

believe that the two pigments were identical with the L xanthophyll which he had isolated in a crude way from flowers and green grass.

Studies published by Palmer and Eckles[7] have shown that the natural yellow pigment that characterizes the milk fat, body fat, corpus luteum, and blood serum of the cow is physiologically, as well as chemically, identical with the carotin of the chloroplast, and depends upon the presence of this pigment in the food for its presence in the body tissue, fluids, and secretions of the animal body. Combining these results with the findings of Schunck, and Willstätter and Escher, a similar relation between the xanthophyll pigment of the egg yolk and the xanthophyll of the chloroplast naturally suggests itself. Should such a result be confirmed there would be presented the very interesting phenomena of the inability of the cow to take up the xanthophyll pigments to any extent and the similar inability of the fowl to make use of the carotin; the major pigment being carotin in the case of the cow and xanthophyll in the case of the hen.

Such a result in the case of the fowl would be especially striking in view of the ease with which it has been found possible to deposit fat-soluble dyes in the egg yolk and body fat, and even transmit them to the chick. Thus, Gage[8] has deposited Sudan III in the egg yolk and body fat of the hen by feeding the dye, and the pigment was also found in the fatty tissue of the newly hatched chicks from eggs colored in this way. Similar results have been obtained by Mendel and Daniels.[9]

Laying aside for the present the question of the relative utilization of the plant carotin and xanthophylls by the hen, a physiological relation between the primary egg yolk pigment and the xanthophylls of the feed is reasonably well established by published observations of the influence of various feeds upon the color of the yolk. Thus the Maryland Agricultural Experiment Station has carried on feeding trials of this character[10] in which yel-

[7] Palmer, L. S., and Eckles, C. H., *Jour. Biol. Chem.*, 1914, xvii, 191; *Missouri Agricultural Experiment Station Research Bulletins*, Nos. 9, 10, 11, and 12, 1914.

[8] Gage, S. H., and Gage, S. P., *Science*, 1908, xxviii, 494.

[9] Mendel, L. B., and Daniels, A. L., *Jour. Biol. Chem.*, 1912-13, xiii, 71.

[10] Opperman, C. L., *Country Gentleman*, 1914, lxxix, pt. i, 432.

low corn, comprising about half the ration and fed as a scratc feed with bran, gluten meal, and beef scrap to laying hens, wa compared with a ration in which the yellow corn was replaced b whole wheat and in another case with equal parts of yellow cor and wheat. The eggs from the lot receiving yellow corn a showed yolks with a deep yellow color; those from the lot recei ing corn and wheat showed yolks with a fair yellow color; whi the eggs from the wheat-fed lot had yolks noticeably very pa colored. The simplest explanation of this result is found in th fact that yellow corn is very rich in xanthophyll, as was pointe out by Palmer and Eckles from this laboratory in connection wit the relation of the milk fat pigment to the plant carotin an xanthophylls.

It was with the view of furnishing definite experimental ev dence of a physiological relation between the plant xanthophyl and the natural egg yolk pigment that the experiments her reported were instituted.

Methods of Investigation.

The several methods by which it was expected to establish th physiological relation between the plant xanthophylls and th egg yolk pigment were as follows:

1. To ascertain whether feeds carrying xanthophyll to th exclusion of carotin will increase the amount of xanthophy carried by the blood serum and deposited in the egg yolk.

2. To ascertain whether feeds free from both xanthophyll an carotin will reduce the amount of xanthophyll carried by th blood serum and deposited in the egg yolk, and to what exten such a reduction, if possible, can be carried.

3. To ascertain whether feeds carrying carotin to the exclusio of xanthophyll will increase the carotin carried by the blood serum and egg yolk; in other words, to ascertain to what extent the laying hen can make use of carotin for the pigmentation of the egg yolk.

4. To ascertain, if possible, how the blood serum of the hen carries the carotin and xanthophyll pigments. The studies with the cow showed that the carotin was carried by the blood serum as a water-soluble caroto-albumin.

Character of Rations and Pigments Fed.

The experimental rations selected to conform to the plan outlined above, together with the ration which the hens received preliminary to the experimental rations, are shown in Table I.

TABLE I.

Preliminary and Experimental Rations.

Preliminary.	Parts.	Non-pigmented.	Parts.	Xanthophyll.	Parts	Carotin.	Parts
Yellow corn,	11.3	White corn,	16.0	Yellow corn,	16.0	White corn,	16.0
Wheat,	4.7	Bran,	2.0	Bran,	2.0	Bran,	2.0
Bran,	2.0	Middlings,	2.0	Middlings,	2.0	Middlings,	2.0
Middlings,	2.0	Beef scrap,	1.0	Beef scrap,	1.0	Beef scrap,	1.0
Beef scrap,	1.0					Carrots,	....

The table shows that yellow corn comprised a little over one-half the preliminary ration and about 75 per cent of the xanthophyll ration.

The rations were fed in the following manner:

Preliminary rations.	Experimental rations.
½ Scratch feed	⅔ Scratch feed
Corn 2 parts	Whole and ground corn
Wheat 1 part	⅓ Mash
½ Mash	Corn 1 part
Corn 1 part	Bran 1 part
Bran 1 part	Middlings 1 part
Middlings 1 part	Beef scrap ½ part
Beef scrap ½ part	

Later in the experiment two changes were made in the rations; the bran was taken out of the non-pigmented and carotin rations as being a source of too much xanthophyll; the scratch feed reduced to one-third in the xanthophyll ration, and a corresponding amount of ground yellow corn incorporated in the mash. The date of these changes is shown in Table III.

In the case of the carotin ration the same proportion of the feeds was used as in the non-pigmented ration, but a smaller amount of total feed was given the hens and they were given access to as much whole and pulped carrots as they would eat. The pulp was mixed with the grain mash and every opportunity

given the hens to consume as large a quantity of the carrots as possible.

A careful study was made at the beginning of the experiment in regard to the amount of pigment carried by the different constituents of the rations, and particularly whether they carried appreciable amounts of xanthophyll. These studies were made by extracting the air dry materials with alcohol and ether until no more yellow pigment was extracted, securing the unsaponifiable pigment in the usual way, and then studying the relative proportion of the total color due to carotin and xanthophyll respectively. This was done by a careful separation of the total pigment between petroleum ether and 80–85 per cent alcohol until each solution yielded no more pigment to fresh portions of the

TABLE II.

Relative Proportion of Color Extracted from Feeds Due to Carotin and Xanthophylls.

Feed.	Xanthophyll.		Carotin.	
	Units of yellow.	Units of red.	Units of yellow.	Units of red.
Bran, middlings, beef scrap mash.....	33.0	0.8	9.0	0.9
Yellow corn.....	60.8	3.8	54.0	1.5
Carrots.....	36.0	1.0	46.8	6.5

other solvent. In this way practically all of the carotin was obtained in the petroleum ether, and the xanthophyll in the alcohol. Each portion of the total pigment was concentrated to volume of 12.5 cc. and the color noted in a one inch layer with the Lovibond tintometer. The amount of feed taken for study was sufficient for one day's feed for two hens, with the exception of the yellow corn and carrots, where just sufficient was used to give a good analysis. Table II shows the results of these studies. Tests on the individual constituents of the mash showed that practically all the carotin came from the beef scrap and the greatest proportion of the xanthophyll from the bran. It was on the basis of this study that the bran was subsequently removed from the ration of the hens on the non-pigmented and carotin diets.

The appreciable quantity of xanthophyll in the carrots is notable. Similarly the yellow corn contained considerable carotin. In interpreting the tintometer readings the units of red must be taken into account as well as the units of yellow, for it is the former that measure the intensity of the pigment. Unfortunately, the color readings do not give a quantitative measure of the pigment. This is due to the fact that carotin is a much more intense pigment than xanthophyll. Using the fact that equal units of red and yellow are together equivalent to a pure orange color, it is seen that under the experimental conditions used the color produced by the xanthophyll of the yellow corn consisted of 3.8 units of orange and 57 units of yellow while the color produced by the carotin of the yellow corn consisted of 1.5 units of orange and 52.5 units of yellow. Thus the xanthophyll solution was only about 8.5 per cent more yellow but over one and one-half times as orange as the carotin solution from the same corn. Similar interpretations are readily made for all the tintometer readings and are particularly significant when applied to the egg yolk colors given in a subsequent table.

Six White Leghorn hens, two in each group, were used for the experiment. All the hens were characterized by having pale yellow shanks and beaks, and colorless ear lobes. Each pair of hens was housed separately throughout the entire experiment on a dirt floor covered with clean straw. The hens never had access to any food other than the experimental rations. All the eggs were saved and the color of the yolks was noted. Several eggs were obtained from each group while still on the preliminary ration.

In studying the color of the yolks the plan was to hard boil the eggs, remove the yolk and spread it out in a small tray before the Lovibond tintometer, and compare the color with the standard glasses viewed from a similar amount of the Lovibond "standare white"[11] spread out in a similar way. As the experiment progressed, however, the increasing paleness of the yolks of the eggs from two of the groups made it necessary to change the method in order that more color could be observed for analysis. The second method consisted in a careful separation of the white

[11] The "standard white" is the highest purity $CaSO_4$.

TABLE III.

Effects of Rations on Color of Egg Yolk.

Non-pigmented ration.			Xanthophyll ration.			Carotin ration.		
Date.	Units of yellow.	Units of red.	Date.	Units of yellow.	Units of red.	Date.	Units of yellow.	U
Preliminary ration.			Preliminary ration.			Preliminary rati		
1915			1915			1915		
Feb.			Feb.			Feb.		
19	4.5	1.1	19	4.0	1.1	17	3.2	
20	4.5	1.0	21	5.0	1.2	17	2.6	
20	5.0	1.2				20	3.2	
22	5.0	1.2				22	2.5	
Experimental ration.			Experimental ration.			Non-pigmented rat		
23	4.5	1.2	23	4.0	1.2	23	2.5	
25	4.0	1.0	24	7.0	1.7	24	1.7	
25	3.7	1.0	26	7.5	1.8	25	2.5	
27	3.3	1.0	26	5.5	1.3	26	5.0	
27	3.5	1.0	27	6.0	1.7	Carotin ration.		
Mar.			28	4.5	1.7	28	3.7	
1	2.7	1.0	Mar.			Mar.		
1	3.0	1.1	1	4.9	1.3	1	3.2	
3	2.4	0.6	1	4.0	1.4	3	2.5	
3	2.0	0.5	2	3.5	0.9	4	2.4	
			2	4.5	1.1			
			4	4.1	1.0			
			4	3.5	0.8			
5*	5.0	1.8	5*	7.0	3.4	6*	6.0	
5	6.0	2.8	6	7.0	3.2	8	5.9	
7	6.0	2.8	7	6.9	3.2	10	5.9	
7	5.0	1.7	8	6.9	3.4	13	4.8	
9	5.9	2.6	8	6.9	2.9	15	6.0	
9	5.0	1.9	10	6.9	3.2	16	5.4	
10	5.9	2.4	12	6.9	3.2	17**	5.4	
12	4.9	2.1	13	7.0	3.2	19	4.9	
13	5.4	1.9	15	7.3	4.0	20	6.1	
14	4.5	1.8	17	5.8	3.3	22	5.4	
16***	4.1	1.6	19	7.8	4.1	28	4.9	
17	5.0	1.5	19	7.3	3.5	29	5.4	
18	4.5	1.5	20	7.3	3.4			
19	4.5	1.5	21	7.8	5.1			
21	4.0	1.5	22	7.8	4.8			
22	4.0	1.3	22	7.8	4.0			
24	3.5	1.2	24	6.8	3.3			
26	3.2	1.0	26	6.8	3.5			
27	3.3	1.0	26	5.8	4.0			
27	4.0	1.5	28	6.8	3.0			
29	4.0	1.2	29	6.8	3.8			
29	3.2	1.0	31	7.8	4.8			

* Raw yolk analysis began.

** One hen of this lot stolen.

*** Bran removed from ration of non-pigmented and carotin lots scratch feed of xanthophyll lot cut to one-third of ration.

and yolk of the raw egg and spreading the raw yolk upon a white porcelain crucible cover.

The experiment was stopped when the egg yolks of the three lots of hens had apparently been influenced as much as the rations used would allow. All the hens were bled to death and studies made in regard to (1) the amount of pigment carried in the blood serum of each group, (2) the character of the pigment present, and (3) the way in which the serum carried the pigment.

RESULTS OF EXPERIMENT.

Influence of Rations on Color of Yolks.

The color of the egg yolks when the hens were on the preliminary ration, and the influence of the different experimental rations on the color as determined by the methods given above are shown in Table III. The maximum effects of the three rations are given in Plate 2.

An examination of the data in Table III shows that a gradual reduction in the amount of pigment deposited in the yolk accompanied the change from a ration carrying a moderate amount of xanthophyll (preliminary ration) to a ration carrying a very small amount of xanthophyll. In eight days the color of the yolks of the non-pigment group had become so pale that the hard boiled yolks showed scarcely enough color to measure with the Lovibond tintometer. Very little further reduction in color occurred until the bran was removed from the ration on March 16. In eight days the color had reached the lowest level attained, the tintometer reading of the raw yolk showing only 3.2 units of yellow and 1.0 unit of red. It is not probable that the color of the yolks of this lot of hens would have been reduced further. The ration still contained a small amount of xanthophyll, certainly sufficient to account for all that was deposited in the yolks when it is considered that the pigment found there represented the accumulated pigment carried in the blood through the period during which the yolk was being formed. According to Rogers[12] the period required for the complete formation of the yolk is

[12] Rogers, C. A., *Proc. Internat. Assn. Instructors and Investigators in Poultry Husbandry*, 1909, i, 77.

about fourteen days. (Observation made during heavy laying season.) It is apparent from this that an absolute elimination of pigment from the ration would be necessary in order to produce an absolutely non-pigmented yolk.

Very interesting results were obtained on increasing the xanthophyll in the ration in the case of the xanthophyll-fed hens. As anticipated, a considerable increase in the color of the yolks accompanied the addition of more xanthophyll. Especially striking, however, was the almost immediate effect of the added xanthophyll as shown by the data in Table III. A marked increase in the color was noted on the second day after the change in ration. This was no doubt due to the fact that the greater part of the yolk is formed during a relatively short period of time. As a matter of fact it was observed in the case of the highly colored yolks from the eggs laid on February 24 and 26 that it was the outer part of the yolks that was more intensely colored. The high color obtained at the beginning of the experiment did not persist, however, but a gradual reduction occurred until the level of the preliminary ration was reached. This was judged to be due to a failure to consume the scratch feed which contained by far the greater proportion of the xanthophyll of the total ration. When this was remedied by reducing the scratch feed to one-third, instead of two-thirds of the ration, and increasing the amount of yellow corn in the mash a corresponding amount (March 16), a rapid rise in the color of the yolks occurred at once, a maximum of 7.8 units of yellow and 5.1 units of red being reached on the fifth day after the change. The pronounced orange tint of the color is notable, the tintometer reading showing 5.1 units of orange and 2.7 units of yellow, nearly twice as much orange as yellow.

The results obtained with the carotin-fed hens were in every respect similar and nearly identical with the results from the hens fed the pigment-free ration. Although the former studies made in this laboratory on the relative utilization of carotin and xanthophyll by the cow anticipated in a measure the results obtained with the hen, it was nevertheless astonishing to find to what a small extent the hen is able to take up the carotin from her feed and deposit it in the egg yolk. The data show that the change of the ration caused a decrease in the color of the yolk.

When the carrots were added to the ration on February 27 there was practically no effect on the color of the yolks. In spite of the fact that the hens in this group were not laying as many eggs as the hens on the non-pigmented ration, which would tend to increase to some extent the color of the yolks, the color analyses of the two groups of hens followed each other almost identically up to the time the bran was removed from the ration of the two groups on March 16. Up to this time it was not possible to tell which eggs came from the non-pigment group and which came from the carotin group. After March 16, however, the egg yolks from the hens fed the pigment-free ration suffered a further decline in color, as already noted, which was not obtained in the case of the carotin-fed hen. This may have been due to the fact that more carrots were eaten after the removal of the bran from the ration, thereby supplying about as much xanthophyll as was removed with the bran. Some of the color deposited in the yolks of this group of hens was unquestionably carotin in nature, as a study of the egg yolk at the end of the experiment showed. It is also probable that a slightly greater proportion of carotin to the total pigment resulted. These studies are reported below. It is impossible, however, for the failure of the carrots to materially increase the color of the egg yolk to have been due to a failure on the part of the hens to consume an adequate amount of the food. Large amounts of carrots were found in the craw and gizzard of the carrot-fed hen that was killed at the close of the experiment.

Influence of Rations on Pigment Carried by Blood Serum.

At the close of the feeding experiment the five hens remaining on the test were bled to death. The blood of each pair of hens was combined, and the blood defibrinated at once by vigorous shaking with glass beads in an Erlenmeyer flask. The defibrinated blood was filtered and centrifuged until perfectly clear. Analyses of the amount of color due to the combined carotin and xanthophyll carried by a unit volume of serum were made on the serum from each experimental group of hens. The method of analysis was to desiccate 5 cc. of the serum with plaster of Paris and shake the powder with ether, and then with petroleum ether,

after moistening with absolute alcohol, until no more color was extracted. The combined extracts were concentrated to small volume, made up to 12.5 cc. with absolute alcohol, and the color of this solution was observed in one inch layer in the Lovibond tintometer.

In the case of the combined blood from Hens 1 and 2, fed the non-pigmented ration, 5 cc. of the serum yielded no perceptible color using the above method. On addition of sufficient alcohol to precipitate the proteins from the total serum remaining (50 cc.) and shaking with a mixture of ether and petroleum ether in a separatory funnel, a noticeable, but faint, yellow color appeared in the ethereal layer. Attempts to secure more of the pigment from the alcoholic layer were without success, although the protein was filtered off and boiled with absolute alcohol, and the extract, in combination with the filtrate from the proteins, carefully extracted with ether after saponification with NaOH. This ether extract was added to the first petroleum ether extract of the serum for fear that it might contain traces of pigment imperceptible to the eye. In order to obtain all the yellow pigment carried by the blood of the two hens, with the exception of the 5 cc. of serum first tested, an extract was made of the total corpuscle layer from the centrifuging of the defibrinated blood. This was done by desiccating with plaster of Paris and shaking with a mixture of equal parts of alcohol and ether. The extract was diluted with much water and the ether layer which rose to the top washed clear with water. It contained a bare trace of yellow color. This was added to the extracts already obtained from the serum, the combined solution containing all the yellow pigment carried by the blood of the two hens on the non-pigmented ration, with the exception of 5 cc. of serum. The pigmented solution was concentrated in absolute alcohol, made up to 12.5 cc. with the same solvent, and the color noted in one inch layer in the Lovibond tintometer. The result is given in Table IV. A relative solubility separation of the pigment between 83 per cent alcohol and petroleum ether showed that a small proportion of the pigment was carotin, but the greater part showed the properties of xanthophyll.

The analysis of the combined blood from Hens 3 and 4, fed the xanthophyll ration, was in marked contrast to that from the hens

the non-pigmented ration. Whereas the blood serum from latter showed no perceptible pigment in 5 cc., and only a ll amount in the total blood, 5 cc. of the serum from the hophyll-fed hens yielded about one-half as much color as was ined from the total blood of the two hens fed the non-pig-ted ration. The results of the analysis of three 5 cc. por-s of the serum, carried out by the method previously described, shown in Table IV.

TABLE IV.

Influence of Rations on Amount of Pigment in Blood Serum.

Ration.	Volume of serum used.	Color of extract.	
		Units of yellow.	Units of red.
	cc.		
hophyll (Sample 1).....	5	7.5	0.5
hophyll (Sample 2).....	5	6.5	0.5
hophyll (Sample 3).....	5	7.0	0.6
pigmented.....	5	No perceptible color.	
pigmented.....	50 (blood)	16.0	1.7
tin.....	5	No perceptible color.	

n examination of the pigment extracted in all three cases the xanthophyll serum showed the presence of a portion rela-y more soluble in petroleum ether than in 85 per cent alco-indicating carotin. In the case of Sample 2, the separation e carotin and xanthophyll was made as quantitative as pos- and the color of each portion observed in 12.5 cc. volume one inch layer. The results were as follows:

portion of Pigment from Serum Due to Carotin and Xanthophyll.

	Units of yellow.	Units of red.
arotin.....	2.0	0.2
anthophyll.....	4.5	0.3

ie examination of the blood serum of Hen 5, fed the carotin n, gave results identical with those obtained from the serum ie hens fed the non-pigmented ration. Two trials with 5 ortions of the serum, extracting in one case with ether and oleum ether after complete desiccation with plaster of Paris,

and in the other case precipitating the proteins with alcohol and extracting the precipitate with boiling alcohol, failed to yield any yellow color perceptible to the eye. There was not sufficient serum remaining from the one hen for further study. Very clearly, however, the amount of pigment carried by the serum in the case of the carotin-fed hen had been reduced to a very low quantity, as in the case of the hens fed the non-pigmented ration.

Transportation of Carotin and Xanthophyll by the Blood.

The studies made by Palmer and Eckles[13] in regard to the transportation of the carotin and xanthophyll in the blood of the cow showed that the carotin is carried as a water-soluble compound of the albumin of the serum, while the xanthophyll, which is present in relatively small proportion, is carried by the fat of the blood. Probably the most striking demonstration of this is seen in the failure to extract the pigment from either the fresh or desiccated (with plaster of Paris) serum with pure ether (free from alcohol); while the addition of alcohol to the serum or to the plaster of Paris mass sufficient to coagulate the proteins, will liberate the carotin so that it may be readily extracted with petroleum ether, the result being identical with the extraction of carotin from 80–85 per cent alcohol with this solvent. Other properties of the carotin in the blood serum of the cow, and the method of isolation of the caroto-albumin are described in the previous report of this investigation.

Similar studies made with the serum from the xanthophyll-fed hens failed to give conclusive evidence of a transportation of the xanthophyll by means of the albumin, although conclusive evidence was obtained that the globulin fraction of the serum is free from the pigment. Further study will be required to determine how hen serum carries the xanthophyll. One marked difference between the properties of the carotin of the cow serum and the xanthophyll of the hen serum was noted, however, which is worthy of mention. Whereas it is possible to extract only traces of the carotin from cow serum by shaking the fresh or desiccated serum with ether, the entire pigment of the hen serum

[13] Palmer and Eckles, *Jour. Biol. Chem.*, 1914, xvii, 229; *Missouri Agricultural Experiment Station Research Bulletins*, No. 12, 1914.

was found to be readily extracted by this solvent from the fresh as well as from the desiccated serum. Both the hen and cow serum were similar, however, in their failure to give up their respective pigments to petroleum ether and carbon bisulphide. The significance of these properties remains to be determined.

The Influence of the Rations on the Relative Proportion of Carotin and Xanthophyll in the Egg Yolk.

The feeding of the high proportion of xanthophyll and carotin respectively in two of the groups of the experiment raised the question whether this would result in changing the proportion of the two pigments deposited in the egg yolk. An actual quantitative measurement of the pigments was, of course, out of the question; but it was thought that an excellent comparison of the effects of the ration in this regard could be obtained by comparing the amount of color due to the two classes of pigment, as was done in the similar studies of the individual feeds constituting the rations. Comparisons were made on the basis of weight of raw yolk equivalent to one egg yolk. For example, in the case of the preliminary ration eggs, the yolks of the seven eggs involved were combined, thoroughly mixed, and weighed, and one-seventh of the weight was taken for the relative proportion analysis. The yolks from the last two eggs laid in the non-pigmented and xanthophyll groups were treated similarly. The analysis thus represents the average from the two hens in the group. In the case of the carotin group, the analysis was made on the yolk of the last egg laid, there being only one hen left in this lot. The method of analysis in every case[14] was to desiccate the material with plaster of Paris and extract the desiccated mass with ether and methyl alcohol until all the pigment was extracted. The extract thus obtained was saponified with 20 per cent methyl alcoholic potash solution; the pigment was recovered from the soap with ether in the usual way, and separated as completely as possible between 83 per cent alcohol and petroleum ether. Each portion was concentrated at once and the color of the solution noted at a volume of 12.5 cc. in one inch layer with the Lovi-

[14] Except in the case of the preliminary ration where the hard boiled yolks were directly extracted with ether until no more pigment was extracted.

bond tintometer. The results obtained, together with the color of the raw yolk, are shown in Table V.

The two striking features of the table are, (1) the relatively greater proportion of color due to carotin in the carotin lot egg than in the non-pigmented lot eggs indicating a somewhat better utilization of carotin when fed in large amounts, and (2) the extremely low total color of the yolks of the eggs from the hens fed the pigment-free ration. This analysis shows in even more striking manner than the figures in Table III the extent to which the yolk pigment was reduced in this experiment.

TABLE V.

Influence of Different Rations on Proportion of Color in Egg Yolk Due to Carotin and Xanthophyll.

Ration fed.	Color of yolk.		Color of pigments of yolk.			
	Units of orange.	Units of yellow.	Xanthophyll.		Carotin.	
			Orange.	Yellow.	Orange.	Yellow.
Preliminary....	3.16	3.84	2.5	44.1	0.8	26.0
Xanthophyll...	3.40	3.40	2.8	58.0	0.5	31.5
Carotin.....	1.70	3.20	0.6	32.4	0.6	8.4
Pigment-free...	1.10	2.50	0.5	5.5	0.2	1.1

The Pigment of the Body Fat of Hens.

The studies made in this laboratory on the yellow pigments characterizing the fatty tissue of the cow showed that they were identical with the pigments of the butter fat, and consisted of carotin and xanthophyll, the former pigment being present in by far the greater proportion. Similarly it was anticipated that the pigment of the fatty tissue of the hen would be found to be identical with the pigment characterizing the egg yolk and blood serum, and consist of carotin and xanthophyll, with the latter pigment in the greater proportion. This was found to be the case. Tissue fat from one of the hens on the pigment-free ration was rendered and the pigment isolated in the usual way from 30 grams of the rendered fat. A careful separation of the total pigment obtained between 83 per cent alcohol and petroleum ether showed that by far the greater part of the pigment was more sol-

le in the alcohol. The total carotin and xanthophyll from the grams of fat gave the following tintometer readings in 12.5 cc. lume, one inch layer:

Proportion of Color of Body Fat Due to Carotin and Xanthophyll.

	Units of yellow.	Units of red.
Carotin from body fat	9.0	0.5
Xanthophyll from body fat	54.0	1.5

Influence of the Rations on the Color of the Body Fat.

Observation of the carcasses of the hens at the close of the ex-iment indicated that the different rations had been without luence upon the body fat. In the case of the hens fed the ıthophyll and pigment-free rations, portions of the caul and

TABLE VI.

Color of Body Fat, Xanthophyll, and Non-pigmented Rations.

	Units of yellow.	Units of red.
-pigment-fed hens:		
en 1	60.0	1.0
en 2	60.0	0.7
thophyll-fed hens:		
en 3	60.0	1.2
en 4	70.0	2.2

ıentery fat were saved, the fatty tissue was rendered, and the ›r of the rendered fat noted in one inch layer with the tintom-r. The results are shown in Table VI.

ılthough the average color of the fat of the xanthophyll-fed ıs was slightly higher than that of the hens fed the pigment-› ration, the difference is scarcely great enough to be signifi-t. This is particularly true inasmuch as there was a wide erence in the amount of tissue fat around the digestive organs :he hens. Hens 2 and 3 had relatively small fat deposits in se regions, while the similar deposits were abundant in the e of Hens 1 and 4.

ıs already noted all the hens began the experimental ration h very pale yellow shanks and beaks. Observation showed t none of the rations had any influence in either increasing or

decreasing the pigment deposits in these places. All the hens were as near alike in this regard at the end of the experiment as they were at the beginning. The failure of the xanthophyll ration to increase the color of the shanks must be attributed to the failure of the ration to cause a deposition of fat in the lower epidermal layers of the shank skin, for it has been shown by Barrows[15] that yellow shanks are caused by yellow fat deposits in the Malpighian layer of the epidermis. It would also seem probable that a deficiency in the normal amount of fat in the shank skin of the xanthophyll-fed hens was also partly responsible for the lack of increase of the shank color. The basis for this view is the repeatedly[16] demonstrated fact that fat deposits already laid down in the fowl are readily stained by feeding fat dyes, such as Sudan III. Whether the natural yellow pigment of the fowl acts in the same manner has not been demonstrated.

DISCUSSION AND APPLICATION OF RESULTS.

As already pointed out, the experiments reported in this paper showing the physiological, as well as the chemical identity of the yellow pigments characterizing the egg yolk and the body fat and blood serum of the hen with the carotin and xanthophylls of plants were anticipated from similar studies involving the pigments of the body fat and blood serum of the cow and the butter fat of the milk. The most significant feature of these results, however, is that the hen utilizes relatively little carotin in the pigmentation of the egg yolk and body fat, the principal pigment belonging to the xanthophyll group of plant pigments. This is exactly opposite to the utilization of the plant carotin and xanthophylls by the cow where carotin is the greatly predominating pigment taken up and transmitted to the mammary gland and other fat synthesizing cells. There is no explanation available at present for the difference in the utilization of these pigments by the hen and cow, outside of the wide difference in the digestive systems of the two classes of animals. Further studies are necessary in order to clear up this interesting phenomenon.

[15] Barrows, H. R., Histological Basis of Shank Colors in the Domestic Fowl, *Maine Agricultural Experiment Station Bulletins*, No. 232, 1914.

[16] Rogers, *loc. cit.* Mendel and Daniels, *loc. cit.*

The practical application of the results herein given is in the control of the color of the flesh (body fat) of poultry and of the yolks of the eggs during the winter season. Where the fancy poultry trade demands colorless flesh in the pen-fattened birds it is merely necessary to select feeds free from carotin and xanthophylls, particularly the latter. The measure of success already attained in feeding practices of this character are to be attributed to the fact that skim milk or butter milk comprise the greater proportion of the ration. These feeds are devoid of both carotin and xanthophyll. Experiments involving the raising and fattening of poultry in which the results of these experiments are being applied in the control of the color of the flesh are now being conducted by the Department of Poultry Husbandry of the Missouri Agricultural Experiment Station. Where the demand is for yellow egg yolks throughout the winter months, these experiments find their application in the use of feeds rich in xanthophyll. Yellow corn is particularly suited for this purpose and is one of the few winter feeds carrying an abundance of the xanthophyll pigment which is carried over into the egg yolk.

SUMMARY.

1. The natural pigment characterizing the egg yolk, body fat, and blood serum of the hen is physiologically identical with the carotin and xanthophyll pigments of plants, with the latter class of pigments present in by far the greater proportion. This is different from the utilization of the plant carotin and xanthophylls by the cow, where the carotin is the predominating pigment found in the milk fat, body fat, and blood serum. Feeding tests with laying hens in which the pigment of the feed was carotin to the relative exclusion of xanthophyll were without appreciable influence upon the amount of pigment carried by the blood serum and deposited in the egg yolk. The feeding of rations relatively free from both carotin and xanthophyll to laying hens resulted in a marked reduction of the amount of this pigment carried by the blood serum and deposited in the egg yolk.

2. The experiments reported find practical application in the control of the color of the flesh (body fat) of fattening poultry, and the control of the amount of natural pigment deposited in the egg yolk.

THE BEHAVIOR OF SOME HYDANTOIN DERIVATIVES IN METABOLISM.

III. PARABANIC ACID.

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Despite frequent investigations the problem of the mechanism of uricolysis still remains unsolved. The attempts to identify those substances, whose formation by oxidative agents from uric acid or the purines is easily accomplished by the organic chemist, as products of the intermediary metabolism of uric acid have failed; nor has the study of these same substances in metabolism thrown additional light on the problem. Among these oxidation products of uric acid may be mentioned allantoin, iminoallantoin,[1] uroxanic acid,[1] alloxan,[2] alloxantin,[2] carbonyl-di-urea,[3] and parabanic acid.

Concerning the fate of parabanic acid in the organism, there exists some confusion in the literature. According to Coppola,[4] parabanic acid passes unchanged into the urine. Lusini,[5] however, in an investigation of the pharmacological properties of alloxan, alloxantin, and parabanic acid, was unable to find more than traces of parabanic acid in the urine after feeding it. Koehne[6] after feeding 2 gm. of parabanic acid to a dog was unable to detect an increased oxalic acid content of the urine and could detect the acid itself in traces only. Hence he concluded that the organism was able to destroy parabanic acid. More recently Pohl[7] in an investigation of the metabolism of oxalic acid, found in one experiment, after the subcutaneous injection of about 0.1 gm. of parabanic acid into

[1] Saiki, T., *Jour. Biol. Chem.*, 1909–10, vii, 263.

[2] Lusini, V., *Ann. d. chim. e farm.*, 1894, xxi, 241; 1894, xxii, 385. Cited by Fränkel, S., Die Arzneimittel-Synthese, Berlin, 2nd edition, 1906, 110.

[3] Henius, K., *Ztschr. f. exper. Path. u. Therap.*, 1911–12, x, 293.

[4] Coppola, cited by Fränkel, *loc. cit.*, 155.

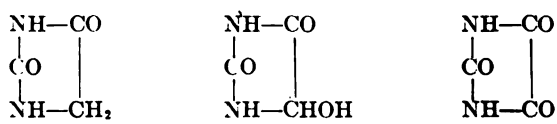
[5] Lusini, *loc. cit.*

[6] Koehne, F., Inaugural Dissertation, Rostock, 1894, 31.

[7] Pohl, J., *Ztschr. f. exper. Path. u. Therap.*, 1910–11, viii, 308.

the dog, 66 per cent of the acid unchanged in the urine. The methods employed in the above mentioned investigations have been of two kinds. Either the isolation and identification of the unchanged acid in the urine have been attempted, or the variations in the oxalic acid content of the urine have been studied. The first method presents many difficulties, as parabanic acid is rather easily soluble and forms no especially typical salts or derivatives. Moreover it is easily broken down in alkaline solution to yield salts of oxaluric acid or urea and oxalic acid, thus requiring extreme care in manipulation to avoid losses. In view of the present unsatisfactory state of our knowledge of the extent to which the organism can oxidize oxalic acid, a study of the oxalic acid content of the urine could hardly be expected to give clear cut results.

In previous studies of this series,[8] it has been shown that the hydantoin nucleus is not attacked by the organism, but is excreted unchanged in the urine. As parabanic acid is readily formed from hydantoin by oxidation with bromine,[9] and is more active chemically and less resistant to hydrolytic agents, it was thought that it might be broken down in the body and a study of its behavior in metabolism was planned.



Hydantoin Allanturic acid Parabanic acid

But since it was found that the nitrogen of parabanic acid was determined by the methods then in use for the determination of urea (Folin, Benedict) and that the problem could not be approached from the standpoint of the urea elimination, on the assumption that parabanic acid would yield *in vivo* as in the laboratory oxalic acid and urea on hydrolysis, further study was discontinued. Recently, however, Van Slyke and Cullen have given us in their modification of the urease method of Marshall, a method specific for urea, which avoids the use of hot concentrated alkaline solutions. After preliminary tests had demonstrated that parabanic acid did not yield its nitrogen as urea nitrogen by the urease method, experiments were undertaken along the lines originally planned.

[8] Lewis, H. B., *Jour. Biol. Chem.*, 1912–13, xiii, 347; 1913, xiv, 245.
[9] Gabriel, S., *Ann. d. Chem.*, 1906, cccxlviii, 50.

Metabolism Experiments.

The animals used were rabbits and a dog. The former were fed a uniform diet of milk and cane-sugar, the food being administered through a stomach sound. The dog was a trained metabolism animal which had been maintained on the low protein diet of the experiment over a long priod of time. The urine was collected at regular twenty-four hour intervals, by catheterization in the case of the dog, and by emptying the bladder by gentle pressure in the case of the rabbits. Nitrogen was determined by the Kjeldahl-Gunning method, and urea by the urease method of Van Slyke and Cullen. The purity of the parabanic acid used was checked by a Kjeldahl nitrogen determination. No toxic symptoms were observed following the administration of the acid, although in one experiment (Rabbit C) the total nitrogen following subcutaneous administration was higher than in a preliminary period.

From the data presented in the table, it is evident that no considerable conversion of parabanic acid into urea has occurred. The increase in total nitrogen eliminated, as compared with the fore and after periods, indicates that the absorption has been nearly complete, although, as was to be expected, not quantitative. No increase in the elimination of urea plus ammonia nitrogen resulted, while an increase in the nitrogen not urea plus ammonia was observed, an increase corresponding to the amount of nitrogen administered as parabanic acid. In the experiment with the dog, a slight increase in the urea elimination took place following the administration of the acid, but as the total nitrogen was also increased above the amount represented by the sum of the average elimination of the fore period and the nitrogen fed as parabanic acid, it is evident that there has occurred a stimulation of metabolism, as a result of the administration of the acid. The increase in the nitrogen not urea plus ammonia nitrogen is in close agreement, as in the other experiments, with the extra nitrogen administered as parabanic acid. No evidence of the conversion of significant amounts of parabanic acid to urea in the organism of the dog or rabbit is to be obtained from the present series of experiments.

Perfusion Experiments.

Sarvonat[10] has reported two experiments in which the liver of a dog was perfused with defibrinated dog blood to which parabanic acid had been added. He concluded from slight increases in the oxalic acid content of the blood after perfusion that parabanic acid is destroyed by the liver. Two perfusion experiments have been carried out[11] in which parabanic acid was added to the perfusion fluid and the urea content of the blood determined by the urease method before and after perfusion.

Dog Z.—Normal male. Weight, 5.04 kg. Weight of liver, 212 gm. 150 cc. blood were diluted to 2,000 cc. with Ringer's solution and 1,800 cc. used for perfusion of the liver. 2 gm. parabanic acid were added to the perfusion fluid. Rate of flow, 504 cc. per minute. Time of perfusion, 1 hour.

Urea nitrogen calculated for total mass perfusion fluid:

	gm.
Before perfusion	0.027
After perfusion	0.057
Difference	+0.030

Dog 95.—Weight, 11.21 kg. Preliminary preparation, 1 gm. phlorhizin in olive oil daily for 4 days. No food. Weight of liver, 446 gm. 320 cc. blood were diluted to 2,000 cc. with Ringer's solution and 1,800 cc. used for perfusion of the liver. 2 gm. parabanic acid were added to the perfusion fluid. Rate of flow, 420 cc. per minute. Time of perfusion, 1 hour.

Urea nitrogen calculated for total mass perfusion fluid:

	gm.
Before perfusion	0.099
After perfusion	0.204
Difference	+0.105

These increases in the urea content of the perfusion fluid, amounting to 64 and 225 mg. of urea in the two experiments, are certainly not great enough to warrant the assumption that the extra urea is formed by the hydrolysis of the parabanic acid added to the perfusion fluid. As shown by Jansen,[12] perfusion of the normal liver with blood diluted with Ringer's solution may result in the formation of urea in amounts comparable to those

[10] Sarvonat, F., *Compt. rend. Soc. de biol.*, 1912, lxxii, 1067.

[11] These experiments were carried out with the aid of Professor A. J. Ringer, to whom I take this opportunity to express my indebtedness.

[12] Jansen, B. C. P., *Jour. Biol. Chem.*, 1915, xxi, 557.

ined in the above experiments. Similar results have been ined by Professor A. I. Ringer[12] in this laboratory, who has observed that the liver of a phlorhizinized dog forms urea readily on perfusion than that of a normal dog, as illustrated ıe present study.

t M. Weight 1,330 gm. Diet: 100 cc. milk and 10 gm. cane-sugar, daily.

	Volume.	Total N.	Urea + NH_3 N.		N not urea + NH_3.	
	cc.	*gm.*	*gm.*	*per cent*	*gm.*	
	75	0.598	0.476	79.5	0.122	
	70	0.492	0.415	84.4	0.077	
	75	0.494	0.389	78.6	0.105	
	100	**0.627**	**0.363**	**57.9**	**0.264**	1 gm. parabanic acid *per os* = 0.246 gm. N.
	65	0.502	0.408	81.3	0.094	
	60	0.568	0.455	80.1	0.113	

t E. Weight 1,000 gm. Diet: 60 cc. milk and 10 gm. cane-sugar, daily.

	Volume.	Total N.	Urea + NH_3 N.		N not urea + NH_3.	
	cc.	*gm.*	*gm.*	*per cent*	*gm.*	
	50	0.281	0.197	70.1	0.084	
	35	0.241	0.155	64.3	0.086	
	105	0.266	0.207	77.8	0.059	
	65	**0.417**	**0.205**	**49.2**	**0.212**	1 gm. parabanic acid *per os* = 0.246 gm. N.
	45	0.340	0.255	75.0	0.085	
	50	0.227	0.176	77.5	0.056	
	35	0.238	0.184	77.3	0.054	

Personal communication from Professor Ringer.

Rabbit C. Weight 1,680 gm. Diet: 100 cc. milk and 10 gm. cane-sugar, daily.

Day.	Volume.	Total N.	Urea + NH_3 N.		N not urea + NH_3.	
	cc.	*gm.*	*gm.*	*per cent*	*gm.*	
1	70	0.501	0.394	78.6	0.107	
2	65	0.537	0.424	79.0	0.103	
3	70	0.495	0.391	79.0	0.104	
4	**80**	**0.720**	**0.430**	**59.7**	**0.290**	1 gm. parabanic acid subcutaneously = 0.246 gm. N.
5	100	0.756	0.613	81.8	0.143	
6	45	0.705	0.538	76.3	0.167	
7	65	0.705	0.525	74.5	0.180	

Dog A. Weight 17.5 kg. Diet: 100 gm. cane-sugar, 40 gm. starch, 50 gm. lard, 40 gm. beef heart, 450 cc. water, daily.

Day.	Weight.	Total N.	Urea + NH_3 N.		N not urea + NH_3.	
	kg.	*gm.*	*gm.*	*per cent*	*gm.*	
1	17.59	2.22	1.67	75.2	0.55	
2	17.59	2.10	1.50	70.1	0.60	
3	17.51	1.96	1.38	71.4	0.58	
4	17.44	2.02	1.44	71.2	0.58	
5	**17.41**	**2.97**	**1.77**	**59.6**	**1.20**	3 gm. parabanic *per os* = 0.738 N.
6	17.40	1.97	1.36	69.0	0.61	
7	17.32	2.04	1.43	70.1	0.61	
8	17.30	1.99	1.37	68.8	0.62	

THE COMPLEX CARBOHYDRATES AND FORMS OF SULPHUR IN MARINE ALGAE OF THE PACIFIC COAST.

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The giant kelps of the Pacific Coast have in recent years received considerable attention because of their high content of potash,[1] possibly of commercial value.[2] The economic aspects of the subject are discussed elsewhere.[3] Of far greater interest to the plant chemist and physiologist is the study of the chemical composition and metabolism of these remarkable plants. Their elective action on certain elements contained in sea water is very striking. Iodine is absorbed in comparatively large quantities from a solution containing only the smallest trace of this element. To a lesser degree there is a marked selective power for potassium. In a previous article the discussion of these points has received further elaboration.[4] It is the purpose of the present paper to present the results of an investigation designed to determine the chemical nature of certain very characteristic organic constituents of several important species of algae growing along the Pacific Coast. The following species are now reported on: *Macrocystis pyrifera*, a brown sea weed belonging to the family

[1] Balch, D. M., On the Chemistry of Certain Algae of the Pacific Coast, *Jour. Ind. and Engin. Chem.*, 1909, i, 777–787.

[2] Cameron, F. K., and Moore, R. B., A Preliminary Report on the Fertilizer Resources of the United States, *U. S. 62nd Congress, Senate Document 90*, 1912, 290 p., 19 plates, maps.

[3] Burd, J. S., The Economic Value of Pacific Coast Kelps, *California Agricultural Experiment Station, Bull. 248*, 183–215, 3 figs.

[4] Hoagland, D. R., Organic Constituents of Pacific Coast Kelps, *Jour. Agr. Research*, 1915, iv, 39–58, 7 tables.

of Laminariaceae;[5] *Iridaea laminarioides;* and *Ulva fasciata.* The two latter are so called rock weeds and do not attain great size. *Macrocystis pyrifera* is noteworthy because of its huge size and is said to have the widest distribution of any known plant.[6]

Studies of the Complex Carbohydrates.

Unlike most land plants marine algae do not, except perhaps in rare instances, contain simple carbohydrates or easily hydrolyzable polysaccharides. Starch is not present, and cellulose only in minor quantities. The most frequently occurring carbohydrates include pentosans, galactans, levulans, and methyl pentosans.

In "Nori" (*Porphyra laciniata*) Kintaro[7] identified as hydrolytic products galactose and mannose. From another species (*Fucus*) Günther[8] obtained fucose. Bauer[9] states that he isolated from one of the Laminariaceae a sugar having the properties of dextrose. Kylin[10] also found in *Laminaria digitata* a carbohydrate yielding dextrose on hydrolysis. He described several other carbohydrates obtained from *Laminaria saccharina, Fucus vesiculosus,* and *Ascophyllum nodosum.* Müther[11] obtained mannitol from *Fucus* and *Laminaria digitata.* According to Euler[12] Carragheen moss (*Chondrus crispus*) yielded galactose, fructose, and a methyl pentose on

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[5] Setchell, W. A., The Kelps of the United States and Alaska, *U. S. 62nd Congress, Senate Document 190,* Appendix K, 1912, 130–178.

[6] McFarland, F. M., The Kelps of the Central Californian Coast, *ibid.,* Appendix M, 1912, 194–208.

[7] Kintaro, O., and Tollens, B., Ueber das Nori aus Japan, *Ber. d. deutsch. chem. Gesellsch.,* 1901, xxxiv, pt. ii, 1422–1424.

[8] Günther, A., and Tollens, B., Ueber die Fucose, einen der Rhamnose isomeren Zucker aus Seetang (Fucus-Arten), *ibid.,* 1890, xxiii, pt. ii, 2585–2586.

[9] Bauer, R. W., Ueber einer aus Laminariaschleim entstehende Zuckerart, *ibid.,* 1889, xxii, pt. i, 618.

[10] Kylin, H., Zur Biochemie der Meeresalgen, *Ztschr. f. physiol. Chem.,* 1913, lxxxiii, 171–197.

[11] Müther, A., and Tollens, B., Ueber die Producte der Hydrolyse von Seetang (Fucus), Laminaria und Carragheen-Moos, *Ber. d. deutsch. chem. Gesellsch.,* 1904, xxxvii, pt. i, 298–305.

[12] Euler-Chelpin, H. K. A. S. v., Grundlagen und Ergebnisse der Pflanzenchemie, Braunschweig, 1908, pt. i, 238 p.

hydrolysis. Takahashi,[13] Saiki,[14] Greenish,[15] and Payen[16] have identified in various species (*Porphyra laciniata, Fucus amylaceus, Fucus evanescens*) pentosans, methyl pentosans, galactans, and mannans.

In all of the studies just referred to the procedure has consisted chiefly in hydrolyzing the sea weed and in establishing the general identity of the sugars thus formed. It would seem to be also of interest to study the properties of the original complexes themselves. This would lead to a better understanding of the chemical composition of the algae. In the present investigation data have been secured regarding the physical and chemical properties of certain well defined complexes in addition to the study of their hydrolytic products.

There are two main fractions of carbohydrates in the algae under consideration, one precipitated from an alkaline extract by acid (so called "algin") and one precipitated by alcohol from aqueous solution. The discussion is accordingly divided into two parts.

Carbohydrates Precipitated by Acid.

The earliest study of this complex, as isolated from sea weeds of the Scottish Coast, was made by Stanford.[17–20] He obtained a jelly-like substance which he called "algin" or "alginic acid." This he characterized as a nitrogenous organic acid, having the structure, $C_{76}H_{76}O_{22}\begin{matrix}NH_2\\NH_2\end{matrix}$.

[13] Takahashi, E., Über die Bestandteile von Fucus evanescens, *Jour. College of Agriculture*, Sappiro, Japan, 1914, vi, pt. v, 109–116.

[14] Saiki, T., The Digestibility and Utilization of Some Polysaccharide Carbohydrates Derived from Lichens and Marine Algae, *Jour. Biol. Chem.*, 1906–07, ii, 251–265.

[15] Greenish, H., Untersuchung von Fucus amylaceus, *Ber. d. deutsch. chem. Gesellsch.*, 1881, xiv, pt. i, 2253.

[16] Payen, M., Sur le gélose et les nids de salangane, *Compt. rend. Acad. d. sc.*, 1859, xlix, 521–530.

[17] Stanford, E. C. C., On Algin: A New Substance Obtained from Some of the Commoner Species of Marine Algae, *Chem. News*, 1883, xlvii, 254–257, 267–269.

[18] Stanford, On Algin, *Jour. Soc. Chem. Indus.*, 1884, iii, 297–301; discussion, 301–303.

[19] Stanford, A New Method of Treating Seaweed, *ibid.*, 1885, iv, 519–520.

[20] Stanford, On Alginic Acid and Its Compounds, *ibid.*, 1886, v, 218–221.

He was unable to wash out the contaminating salts, and evidently worke with a sample only slightly purified.

Krefting, [21, 22] experimenting on the sea weeds of Norway produced "tang acid" similar to the alginic acid of Stanford, but claimed that hi preparation was nitrogen free. Villon[23] and Kylin[10] later described simila substances obtained from other sea weeds, indicating a wide distributio in plants from different localities. An entirely analogous complex is foun in the kelps of the Pacific Coast,[4] approximately 16–18 per cent of tl crude substance in the case of *Macrocystis pyrifera*.

Preparation of Sample.—The preparation of a purified alg free of ash and organic impurities was difficult. The colloid jelly absorbs a large quantity of dissolved organic and inorgan contaminating substances. The purest alginic acid prepar by Stanford[20] and used in determining the molecular formu contained 2.3 per cent ash and 2.03 per cent nitrogen.

The following procedure was adopted in preparing the samp used in the present work. 1 kg. of crushed *Macrocystis* is cover with a 2 per cent Na_2CO_3 solution for twenty-four hours. Tl mixture becomes thick and sticky. It is finally warmed a filtered through linen by suction. The addition of a slight e cess of HCl produces a white spongy precipitate, floating in tl liquid. The color presently darkens to a deep brown. Tl precipitate is filtered off, redissolved in 2 per cent Na_2CO_3, a the precipitation twice repeated. The final precipitation made from an alkaline solution by addition of alcohol. Sodiu alginate comes down as a stringy, non-gelatinous mass, and w keep indefinitely, preserved in alcohol. 1 kg. of dried *Macrocyst* yielded 160 grams of crude alginate, containing 33 per cent as

For final purification the sodium alginate, prepared as describ above, is dissolved in water and placed in a parchment bag i mersed in running water. After three days the solution is acic fied with HCl, which precipitates the alginic acid. The dialy is continued for a few more days in tap water and finally in di

[21] Krefting, A., An Improved Method of Treating Seaweed to Obta Valuable Products (Alginic Acid, "Tang Acid") Therefrom, *Eng. Pa* 1896, 11,583, abstract in *Jour. Soc. Chem. Indus.*, 1896, xv, 720.

[22] Krefting, An Improved System or Apparatus for Treating Seawee (Alginic Acid) for the Manufacture of Products Therefrom, *Eng. Pat* 1898, 12,416, abstract in *Jour. Soc. Chem. Indus.*, 1898, xvii, 846.

[23] Villon, A. M., On "Algine," *Chem. News*, 1893, lxviii, 311.

tilled water until no test for chlorine is given. The alginic acid is then filtered off, dried at 100° C., and finally ground and dried to a constant weight. Samples thus prepared were free of more than traces of ash and nitrogen.

Properties.—Alginic acid is capable of absorbing 200 to 300 times its weight of water. When moist it is readily soluble in dilute alkali, but dried it becomes hard and horny and very resistant to solvents. It is readily precipitated from solution by alcohol and ether. As a colloid alginic acid may be considered an irreversible gel. It is capable of absorbing salts to the extent of 60 per cent of its own weight, but has no selective action for potassium. Its optical activity is high. $[\alpha]_D^{18.5} = -169.2°$. The index refraction is low; $[N]_D^{20} = 1.3373$, for 1 gram of sodium alginate in 100 cc. of solution.

Metallic Derivatives.—A large number of metallic alginates may be formed as described elsewhere.[4] Twenty insoluble and five soluble alginates were prepared by the addition of metallic salts to solutions of sodium alginate, slightly acidified with acetic acid.

Acidity.—Samples of the dialyzed alginic acid were titrated with 0.01 N Na_2CO_3; 325 grams were neutralized by 1 liter of normal alkali. The neutralization equivalent is therefore 325. This result indicates the weak acidity of the substance.

Decomposition of Algin.—Stanford[17] stated that algin was decomposed after several days' standing in dilute alkaline solution. Preliminary work suggested a loss from chemical, bacterial, or enzymic action. Experiments proved that after some time considerable decomposition might take place as a result of bacterial action.

Analytical Data.—Samples of ash- and nitrogen-free alginic acid gave the following data.

	per cent
Furfural calculated as pentosan	23.8
Methyl furfural calculated as methyl pentosan	4.1
Cellulose derivative (4 hours' heating with 1:1 HNO_3)	18.1
Reducing sugars after hydrolysis as dextrose	32.8
Sulphur	None.

The cellulose derivative gave the amyloid test.[24]

[24] Abderhalden, E., *Biochem. Handlexikon*, 1911, ii, 220.

Molecular Formula.—Combustions indicated the following ultimate composition: C = 42.0, H = 4.5, O = 53.5 per cent. The simplest corresponding empirical formula is $C_{21}H_{27}O_{20}$ with molecular weight of 599. Analyses were made of compounds of alginic acid with uni- and divalent metals. The results, using the formula given above, indicate two replaceable H atoms, $H_2(C_{21}H_{25}O_{20})$.

Percentages of Metals in Metallic Alginates.

	Found. *per cent*	Calculated. *per cent*
Na in sodium alginate $Na_2(C_{21}H_{25}O_{20})$.....	7.0	7.1
K in potassium alginate $K_2(C_{21}H_{25}O_{20})$.....	11.2	11.5
Ca in calcium alginate $Ca(C_{21}H_{25}O_{20})$.....	6.3	6.3
Fe in ferrous alginate $Fe(C_{21}H_{25}O_{20})$.....	8.7	8.5

The formula here advanced is obviously only an empirical one. No data are obtainable which would throw light on the manner in which the sugars are linked together. It may be said, however, that alginic acid, if not strictly speaking a definite chemical compound, is at least a homogeneous complex, which shows characteristic reactions.

Identification of Sugars.—Freshly precipitated alginic acid, hydrolyzed with 2 per cent HCl for four hours at 100°C. gave a strong reducing action. Considerable carbonization took place. To avoid this digestions were made for twenty-four hours at 80° C. The undissolved residue was filtered off and the filtrate neutralized with NaOH. The solution was then heated with a mixture of two parts phenylhydrazine hydrochloride and three parts sodium acetate, according to the method of Fischer.[25] Two osazones separated out, a yellow osazone crystallizing readily, and in lesser quantity a red amorphous form. After repeated crystallizations from 50 per cent alcohol, the separation and purification of the yellow crystalline osazone was accomplished. Under the microscope fibrous needles, characteristically arborescent, were observed. The melting point of the crystals was 154–155°C. By comparison *l*-arabinose phenylosazone[26] has a melting point

[25] Fischer, E., Verbindungen des Phenylhydrazins mit den Zuckerarten, *Ber. d. deutsch. chem. Gesellsch.*, 1884, xvii, pt. i, 579–584.

[26] Abderhalden, *loc. cit.*, 288.

of 160°C. and *l*-xylose phenylosazone[27] 152–155°C. Optical activity was determined according to the method of Neuberg.[28] 0.2 gram of the pure osazone was dissolved in 4 cc. pyridine and 6 cc. absolute alcohol, then polarized in a 100 mm. tube, $[\alpha]_D^{20} = -0° 10'$. Under the same conditions for *l*-arabinose phenylosazone $[\alpha]_D^{20} = +1° 10'$ and for *l*-xylose phenylosazone $[\alpha]_D^{20} = -0° 15°$.

Solubilities.—The yellow osazone from the alginic acid is soluble in cold and hot water, benzene, ligroin, alcohol, ether, acetone, chloroform, and pyridine. *l*-Arabinose phenylosazone is insoluble in ether. *l*-Xylose phenylosazone has solubilities similar to those of the osazone prepared from algin. The latter has a crystalline structure distinctly different from that of arabinosazone. While the pentosazone here described is similar in many properties to *l*-xylose phenylosazone, their identity cannot be positively asserted from data available. Very few pentoses have been isolated from plants and only three so completely described as to make identification certain.[23,27]

Carbohydrates Precipitated by Alcohol.

The alcohol-precipitable fraction of the carbohydrates of marine algae has not received attention from previous investigators. In *Macrocystis pyrifera* there is present approximately 11 per cent of alcohol-precipitable matter in the stems, and 6 per cent in the leaves. *Iridaea sp.* contains about 13 per cent.

The method of preparing the samples was the following. A kilogram of the crushed sea weed is first extracted cold with 2 per cent HCl. The liquid is then pressed from the sea weed and filtered. Strong alcohol is added to the filtrate and causes a light flaky mass to precipitate out and settle to the bottom, as a compact cream-colored layer. After a few days the supernatant liquid is drawn off, the precipitate washed with alcohol, and freed of liquid by suction. This precipitation is repeated twice. The final precipitate is preserved under alcohol.

[27] Abderhalden, *loc. cit.*, 297.

[28] Neuberg, C., Ueber die Reinigung der Osazone und zur Bestimmung ihrer optischen Drehungsrichtung, *Ber. d. deutsch. chem. Gesellsch.*, 1899, xxxii, pt. iii, 3384–3388.

Properties.—When dried the alcohol-insoluble matter darkens and becomes sticky in the presence of moisture. The dried material is resistant to solution and to the action of dilute acids and alkalis.

The alcohol-insoluble matter from *Macrocystis pyrifera* is precipitated partially by salts of Co, Zn, Sr, Sn, Cd, Ni, Al, Cr, and Cu. Complete precipitation is effected by ferric chloride, lead acetate, and lead subacetate. The alcohol-insoluble fraction from *Iridaea* is pure white, gelatinous, and gives no precipitate with metals except ferric chloride, lead acetate, and lead subacetate. The evidence in these cases points to complex mixtures of several compounds.

Sulphur Content.—An attempt was made to remove the large amount of inorganic elements found in the substance precipitated by alcohol. A clear water solution was made and dialyzed in parchment for six weeks. At the end of that period there still remained 35 per cent ash ($CaSO_4$) in the preparation from *Macrocystis* and 24 per cent in that from *Iridaea*. No precipitate, however, could be obtained by adding $BaCl_2$ to the aqueous solutions. This would point to the absence of the SO_4 ion. It might be assumed that the Ca and SO_4 are held in organic combination or else in some colloidal complex. After hydrolysis SO_4 could be precipitated directly, as well as the Ca.

Carefully dialyzed samples were dried *in vacuo* and the total sulphur was determined after peroxide fusion.[29] Other samples were burned and the ash was analyzed. The following data were obtained.

	Macrocystis preparation. *per cent*	Iridaea preparation. *per cent*
Total sulphur	13.00	6.91
Sulphur in ash	8.00	4.85
Sulphur volatilized on burning	5.00	2.06

The ash corresponds to a pure $CaSO_4$.

Identification of Sugars.—Acid hydrolysis yielded solutions having a strong reducing action. Determinations were made by

[29] Folin, O., On Sulphate and Sulphur Determinations, *Jour. Biol. Chem.*, 1905–06, i, 131–159.

ı's modification of Fehling's solution and also estimations
e pentoses and methyl pentoses according to the method
llens and Ellett.[30] The results indicated that nearly all the
ing action in the case of *Macrocystis* was due to a methyl
se, while in the case of *Iridaea* no tests for pentose or methyl
se could be obtained from the alcohol precipitate.
e hydrolyzed solution from *Macrocystis* was treated with
rlhydrazine hydrochloride and sodium acetate. Yellow
nes precipitated out on cooling. Three different crystalline
ı were distinguishable under the microscope, including two
-like forms to a comparatively small extent. Almost the
ı mass was made up of an osazone resembling in general
ture arabinose phenylosazone prepared from gum arabic,
ugh the grouping of the crystals was somewhat different.
ated recrystallization from 50 per cent alcohol gave pure
als showing no variation in melting point on further crystalli-
ı. The melting point is 172–173°C. The melting point of
e phenylosazone[31] is 177° C. $[\alpha]_D^{20}$ of the crystals by Neu-
ı method was 0° 0′. They are insoluble in cold or hot toluene
ılcohol. The melting point and solubilities closely resemble
ɔrresponding properties of fucose phenylosazone. Fucose is
nly methyl pentose so far found to occur in marine algae.[31]
ıs prepared from *Laminaria digitata*,[8] *Fucus vesiculosus*,[30]
Nori (*Porphyra laciniata*)[7] by Tollens and his colleagues.
ɛ hydrolysis of the preparation from *Iridaea* was accom-
d as previously described, and phenylosazones were pre-
. The crystals obtained were identical with galactose
rlosazone crystals prepared at the same time.

	°C.
. P. pure crystals	187–188
. P. galactosazone	188
$]_D^{20}$ of pure crystals	+0° 46′
$]_D^{20}$ of galactosazone	+0° 48′

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illett, W. B., and Tollens, B., Ueber die Bestimmung der Methyl-
ɜane neben den Pentosanen, *Ber. d. deutsch. chem. Gesellsch.*, 1905,
i, pt. i, 492–499.
bderhalden, *loc. cit.*, 301–309.

The solubilities correspond to those of *d*-galactosazone.[32] Muc acid crystals were readily obtained and the hydrolyzed sug solution gave Tollens' reaction for galactose.[33] There is litt doubt that the sugar in question is *d*-galactose.

Forms of Sulphur in Algae.

Sulphur is a common and important constituent of mari algae.[4] The analyses of Peterson[31] made on land plants indica much less total and organically combined sulphur than is fou in the algae investigated. This condition is not astonishing wh it is recalled that the sea water nutrient solution contains ve large quantities of soluble sulphates, as compared with the s solution. Especially noteworthy for their sulphur content a the forms *Iridaea* and *Ulva fasciata*. The former has 8.2 per ce total sulphur, the latter 4.4 per cent. A detailed study of t forms of sulphur present was made for *Ulva fasciata*.

Total sulphur (a) was determined by fusion with sodium pero ide, and inorganic sulphur (b) by leaching the sample with wat until no further test for SO_4 was given. Precipitations were ma in each case by Folin's[29] method. Total sulphur was also det mined in the leached residue and in the ash. The difference b tween these two percentages represents the sulphur lost on bur ing. Volatile sulphur was determined by steam distillation the presence of 3 per cent HCl. The distillate was passed in bromine water, which was later evaporated to 2–3 cc., fus with sodium peroxide, and sulphur estimated in the usual wa The other fractions were obtained as indicated in the followi table showing the distribution of sulphur.

Distribution of Sulphur in Ulva fasciata, Per Cent of Dried Material.

a. Total sulphur	4.44
b. Soluble sulphates, calculated to sulphur	2.85
c. Soluble organic sulphur (not precipitated by $BaCl_2$)	.36
d. Total soluble sulphur	3.21

[32] Abderhalden, *loc. cit.*, 349–357.

[33] Hawk, P. B., Practical Physiological Chemistry, Philadelphia, 4 edition, 1913, 41, illus.

[34] Peterson, W. H., Forms of Sulphur in Plant Materials and Their Vari tion with the Soil Supply, *Jour. Am. Chem. Soc.*, 1914, xxxvi, 1290–1300.

e. Insoluble sulphur, not volatilized on burning..... 0.69
f. Insoluble sulphur, volatilized on burning..... 0.47
g. Total insoluble sulphur..... 1.16
h. Sulphur, volatile with steam..... 0.11

Sulphur was observed to be lost on drying the sample at 105°C. This is relatively a small loss and is probably the same fraction which is volatilized by steam distillation. Determinations were made of total sulphur before and after drying. Closely agreeing duplicates yielded the following results.

Sulphur Volatilized at 105°C. (24 Hours).

(Calculated on air dried samples.)

	Total S. Before drying. *per cent*	After drying. *per cent*	Loss of S. *per cent*
Macrocystis pyrifera.....	1.20	1.08	0.12
Iridaea Laminarioides.....	8.97	8.76	0.21
Ulva fasciata.....	4.49	4.36	0.13

Steam distillations were made on the three plants named above and in each case sulphur compounds were fixed in the distillates by bromine water. When the residues from the distillates were being fused with sodium peroxide an odor like that of mustard oil was noted.

SUMMARY.

1. The carbohydrates of *Macrocystis pyrifera* and *Iridaea laminarioides* were investigated and several complex polysaccharides described in detail with reference to their physical and chemical properties.

2. From the acid-precipitable complex known as "algin" a pentosazone, closely resembling *l*-xylosazone, was prepared in pure form and its properties were determined.

3. In the alcohol-insoluble carbohydrate fraction of *Macrocystis pyrifera* a methyl pentose, having the properties of fucose, was described. *Iridaea laminarioides* from a similar fraction yielded only galactose.

4. The high content of sulphur in marine algae, as typified by *Ulva fasciata*, was studied, and estimations were made of the sulphur held in various forms.

EXPERIMENTS UPON THE FATE OF INGESTED SODIUM NUCLEATE IN THE HUMAN SUBJECT.

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Our studies of purine metabolism in the lower mammals[1] having naturally excited our interest in the many unsettled questions connected with the purine metabolism of man, we performed nearly two years ago some preliminary experiments upon the fate of sodium nucleate ingested by ourselves. These experiments, it was intended, should form the starting point of a more extended investigation and if we have decided to communicate these as they stand, it is because circumstances have meanwhile rendered the prosecution of our original plan, at least for the present, impossible. Reported experiments upon the feeding of nucleic acid to the healthy human subject are comparatively few in number. Ours, therefore, if they possess no other value, may deserve record as an addition to the statistics of that particular procedure.

Methods.

Each of us placed himself upon a constant diet so selected as to be adequate, adapted to individual taste, and as far as possible free from purines. To this diet, after an interval sufficient for the establishment of equilibrium, there was added on one day a weighed amount of a sodium nucleate preparation, the purine base content of which had been previously determined. With the first subject (A. H.) three such experiments, with increasing quantities

[1] Hunter, A., and Givens, M. H., *Jour. Biol. Chem.*, 1912–13, xiii, 371; 1914, xvii, 37. Hunter, A., *ibid.*, 1914, xviii, 107. Hunter, A., Givens, M. H., and Guion, C. M., *ibid.*, 1914, xviii, 387. Hunter and Givens, *ibid.*, 1914, xviii, 403.

of the nucleate, were performed at appropriate intervals; with the second (M. H. G.) only one experiment was made. With each the experimental day began and ended at 8 a.m. The urine was collected daily; the feces were collected in short periods marked off by the ingestion of carmine. The purines of the former (uric acid and bases) were determined by the method of Krüger and Schmid;[2] those of the latter by Krüger and Schittenhelm.[3] Whenever the possibility existed that the urine might contain purine compounds more complex than the free bases, the analysis was carried out not only on the native urine but also on a sample which had been boiled several hours with 3 per cent sulphuric acid; the results of this procedure were controlled by applying it also to a number of "normal urines," chiefly from the first subject (A. H.). With this subject we tested also the applicability to human urine of the plan for determining uric acid and bases which we have already employed with the monkey;[4] and we found that the following scheme may in certain circumstances present advantages over the regular Krüger-Schmid procedure.

The copper purine compounds from one-fifth of the day's urine are boiled with dilute HCl (1 cc. of concentrated acid in 200 cc. of water), and decomposed with H_2S. The H_2S is boiled off, the solution filtered with suction, the filter thoroughly washed with boiling water, and the filtrate made up either to 500 or 1,000 cc., according to the amount of uric acid present. The solution is cooled rapidly to room temperature, the volume is finally adjusted, and, before any uric acid has crystallized out, 5 or 10 cc. are taken for the colorimetric determination of uric acid according to Folin and Macallum.[5] The solution is then evaporated to small bulk, the separating uric acid is filtered off, and in the filtrate the bases are determined according to Krüger and Schmid.

The only drawback of this scheme is the time occupied in the evaporation of the large bulk of liquid which is required to keep all the uric acid of human urine, even for a short time, in solution. Its advantages, under most circumstances more than compensatory, are that it disposes of the necessity of weighing or Kjeldahl-

[2] Krüger, M., and Schmid, J., *Ztschr. f. physiol. Chem.*, 1905, xlv, 1.
[3] Krüger, M., and Schittenhelm, A., *ibid.*, 1905, xlv, 14.
[4] Hunter and Givens, *Jour. Biol. Chem.*, 1914, xvii, 37.
[5] Folin, O., and Macallum, A. B., *ibid.*, 1912-13, xiii, 363.

ing the uric acid, and at the same time avoids the uncertainty incident to the use of a correction for solubility.[6] A reference to Table I will show that its results are generally within 5 per cent of those yielded by a nitrogen determination on the uric acid crystals.

The phosphotungstic color reaction, when developed in the product of copper precipitation, yields a beautifully clear blue solution of precisely the same tint as that given by a pure solution of uric acid. The colorimeter readings can therefore be made with a high degree of accuracy. We take, however, this opportunity of stating that we have found the technique of the colorimetric method by no means so free from difficulties as the published descriptions of its authors might make it appear. The experience of this laboratory in fact substantiates in general the criticisms recently made by Benedict and Hitchcock.[7] Particularly have we found these criticisms justified in relation to the proposed standard solution of formaldehyde-uric acid. This exhibited in our hands very wide and apparently quite erratic variations in titer. Their cause, discovered by Benedict and Hitchcock to be the fluctuations of laboratory temperature, escaped us; but in practice they forced us, after repeated trials, to abandon the standard as altogether unreliable. The standard we finally adopted for our own use was an aqueous solution of uric acid, slightly acidified with acetic acid, of such strength that about 1 mg. was contained in 25 cc. This was carefully standardized against a fresh solution of 1 mg. uric acid in 1 cc. dilute lithium carbonate solution prepared as Folin and Denis direct. When preserved by an antiseptic (sodium fluoride was used) it suffered no appreciable deterioration in several weeks. Its principle was of course the same as that of the solution proposed by Benedict and Hitchcock; the latter has the advantage of being five times more concentrated.

[6] We have frequently, when employing the technique of the Krüger-Schmid method, taken occasion to determine colorimetrically the amount of uric acid actually remaining in the filtrate from the separated crystals. The quantities found in one series were 3.9, 9.0, 3.6, 4.7, 3.6, and 6.6 mg. of uric acid. The correction adopted by Krüger and Schmid is 3.5 mg.

[7] Benedict, S. R., and Hitchcock, E. H., *Jour. Biol. Chem.*, 1915, xx, 619.

Experiments with A. H.

The first subject of experiment (A. H.) was 37 years of age, and weighed at the beginning of the record 50.5, at the end 51.2 kg. The diet made use of was the following:

Milk	900 cc.
Egg (boiled)	100 gm.
Cheese	50 "
Bread	350 "
Butter	70 "
Apple	100 "
Sugar	40 "
Infusion of "Instant Postum"	600 cc.
Water	250 "

This diet was calculated (largely from analyses of our own) to contain 13.9 grams of nitrogen, and to possess an energy value of 2,782 calories. It was instituted five days before the collection and analysis of urine were commenced, and was maintained for 21 days thereafter. On the 4th, 9th, and 15th days of the record there were performed Experiments I, II, and III, consisting of the ingestion of sodium nucleate in quantities which are indicated in Table I. The nucleate was not taken in a single dose, but in three approximately equal portions immediately before breakfast (8 a.m.), lunch (1 p.m.), and dinner (6.30 p.m.) respectively. By this procedure it was hoped to render the absorption of the purines very gradual, and so to escape the possible danger of disturbing normal enzymatic processes by the sudden influx of an excess of material. Each fraction of the dose was dissolved in about 100 cc. of water. The urine was analyzed immediately after collection. Its uric acid was determined both according to Krüger-Schmid, and by the adaptation of the colorimetric method already described. Bases were estimated (at least for the first 15 days) after, as well as before, hydrolysis of the urine.[8] The feces, although collected, could not in this case be analyzed in the time at our disposal.

[8] In the hydrolyzed urine we determined, as a matter of fact, uric acid as well as bases. The results generally agreed closely enough with those obtained on the fresh urine. Now and then they were somewhat lower, as if the operation of boiling with dilute acid had destroyed some of the uric acid. We have not thought it worth while to complicate the table by reporting these results.

The results of the analyses are shown in Table I.

The sodium nucleate employed in these experiments contained 8.68 per cent of purine nitrogen. The amount of purine nitrogen ingested was therefore 0.276 gram in Experiment I, 0.525 gram

TABLE I.

No. of experiment.	Day.	Urine.						Remarks.
		Volume.	Total nitrogen.	Uric acid nitrogen.		Purine base nitrogen.		
				Krüger-Schmid.	Colorimetric.	Before hydrolysis.	After hydrolysis.	
		cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
	1	1,240	12.07	0.113	0.107	0.011	0.017	
	2	1,000	12.48	0.117	0.111	0.010	0.016	
	3	1,000	12.06	0.110	0.108	0.009	0.016	
I	4	1,490	13.24	0.161	0.159	0.010	0.018	3.18 gm. sodium nucleate.
	5	1,360	11.88	0.130	0.134	0.011	0.018	
	6	1,230	12.54	0.115	0.119	0.009	0.017	
	7	1,210	11.81	0.110	0.118	0.012	0.017	
	8	1,280	12.24	0.111	0.108	0.011	0.018	
II	9	1,500	13.10	0.147	0.142	0.012	0.017	6.05 gm. sodium nucleate.
	10	1,180	12.36	0.140	0.142	0.012	0.016	
	11	1,450	13.47	0.117	0.114	0.012	0.017	
	12	1,080	12.54	0.119	0.115	0.011	0.016	
	13	940	12.61	0.113	0.122	0.011	0.018	
	14	1,190	12.31	0.111	0.107	0.011	0.017	
III	15	1,380	13.13	0.180	0.179	0.011	0.016	9.12 gm. sodium nucleate.
	16	1,400	13.38	0.160	0.161	0.011		
	17	900	12.44	0.135	0.137	0.009		
	18	1,310	12.20	0.127	0.135	0.011		
	19	1,200	12.33	0.115	0.121	0.011		
	20	1,430	12.95	0.116	0.118	0.009		
	21	1,380	13.24	0.116		0.010		
	22	1,550	10.88	0.093		0.016		Fasting.

in Experiment II, and 0.792 gram in Experiment III. Since the purine content of the feces was not ascertained, it is impossible to be certain what proportion of these quantities was absorbed. We shall assume that the absorption was complete. In the case

of M. H. G., reported below, less than 10 per cent of the purin nitrogen ingested reappeared in the feces; this is in accord wit the usual experience in such experiments. It is therefore unlikel that the assumption made will involve any considerable error.

The administration of sodium nucleate was without effect o the purine bases of the urine. This is true whether these be d termined in the native urine or after boiling with dilute sulphur acid. The latter operation produces an increase in the amount basic nitrogen precipitable by copper, indicating (if we are t rely implicitly upon the method) the presence of complex purin compounds (nucleoprotein, nucleoside, etc.?) but these con pounds, if they really exist, are evidently normal constituents urine, and their amount is not increased by the ingestion of n cleate. As far as the purine nucleus reappears at all in the urin it takes in the present experiment the form of uric acid.

In Table I there are (excluding the day of fasting) thirtee days on which the uric acid output may be confidently assume to be unaffected by exogenous factors. The lowest figure for ar of these days is 0.110, the highest 0.119, and the average of 0.114 gram of nitrogen.[9] On the basis of that average the ext uric acid nitrogen resulting from the administration, in Expe ment I, of 0.276 gram of purine nitrogen is 0.063 gram. This distributed over two days' urine, but as the last fraction of t dose was not taken till 6.30 p.m., it is possible that eliminati was complete within twenty-four hours or less of ingestion. the exogenous purine had been wholly converted into uric aci it would have yielded in that form four-fifths of 0.276, *i.e.*, 0.2 gram of nitrogen. The amount actually recovered represen therefore but 28.5 per cent of the possible. The remainder of t ingested purine is unaccounted for.

The extra uric acid nitrogen in Experiment II amounted (aga in two days) to 0.059 gram. Adopting the same basis of calc lation as before, we find this to be only 14 per cent of a possib 0.420 gram. This is but half the yield of Experiment I.

In Experiment III, where 9 grams of nucleate were taken, t uric acid output remains unmistakably above normal for as mu as four days. This is contrary to the usual experience in su

[9] The figures used in the discussion are those obtained by the Krüge Schmid method.

experiments. It is of course possible that the delayed elimination in the present instance was pathological in character. It is at least worthy of note that on the 19th, 20th, and 21st days (*i.e.*, several days after the ingestion of the nucleate, and when the excretion of the products had apparently come to an end) the subject suffered what was to him an entirely novel experience in the form of "rheumatic" pains localized in the third and fourth metacarpals and corresponding phalanges of the right hand. However, a delay equally great was observed also in the experiment upon the subject M. H. G. (see below), in whose case it was accompanied by no abnormal manifestations of any kind. It would seem therefore that the elimination of uric acid derived from exogenous sources may on occasion normally take place much less promptly than is usually supposed. The total output of exogenous uric acid nitrogen after Experiment III was 0.146 gram, which is 23 per cent of the theoretically possible 0.634 gram.

Attention is called in passing to the fact that a day's fasting lowered the endogenous uric acid output at once by about 20 per cent.

Experiment with M. H. G.

The second subject, M. H. G., age 25, weight 68.0 kg., was placed on the following diet.

Milk	950 cc.
Egg (boiled)	190 gm.
Cream cheese	40 "
Butter	60 "
Bread	60 "
Biscuits ("Uneeda")	40 "
Potato (baked)	100 "
Rice (boiled)	100 "
Sugar	10 "
Jam	40 "
Orange	245 "
Water	ad libitum.

This diet, containing 13.56 grams of nitrogen and 2,450 calories, was instituted several days before analyses were begun. On the fourth day of the actual record 7 grams of sodium nucleate, dissolved in about 250 cc. of water, were ingested at 10.30 a.m.

This forms Experiment IV, of which the result is exhibit Table II.

The 7 grams of sodium nucleate administered on the f day contained 0.608 gram of purine nitrogen. Of this 0.057 appeared in the feces. The amount absorbed was therefore gram, capable of yielding 0.440 gram of uric acid nitrogen. daily output of endogenous uric acid nitrogen for M. H. G. be set at 0.118 gram, the average of the first three days. output on the day of nucleate administration exceeded th

TABLE II.

No. of experiment.	Day.	Urine.						Purine nitrogen of feces.	Remar
		Volume.	Specific gravity.	Total nitrogen.	Uric acid nitrogen.	Purine base nitrogen.			
						Before hydrolysis.	After hydrolysis.		
		cc.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
	1	1,800	1.015	13.12	0.116	0.014			
	2	1,300	1.018	13.06	0.112	0.013	0.021	0.064	
	3	1,400	1.016	13.06	0.125	0.014		"	
IV	4	1,560	1.016	13.09	0.181	0.013	0.020	0.120	7 gm. s nuclea
	5	1,375	1.018	13.96	0.165	0.007		0.062	
	6	920	1.026	12.44	0.141	0.014		"	

0.063 gram, on the next day by 0.047 gram, and on the next by 0.023 gram. The slowness with which the exogenous uri was eliminated has already been referred to. It is possible even on the fourth day some extra uric acid might have be covered if the urine had been collected. The total yield fc three days was 0.133 gram, which is 30 per cent of the the cally possible.

As in the experiment with A. H. none of the ingested p appeared in the urine as free purine bases, and there is no evi that any of it was excreted as bases in combination.

REMARKS.

It is still a matter of debate whether uric acid constitutes in man an intermediate or a terminal product of metabolism. Upon this fundamental question our experiments by themselves offer a very slender basis for debate. Yet there is at least one argument which they may fairly be used to emphasize. That argument depends on a comparison of the results, as recorded in the literature, of all experiments of the same kind as ours, *i.e.*, experiments in which sodium nucleate (or nucleic acid), with its purine content accurately ascertained, has been administered to healthy, or fairly healthy, individuals not at the moment under the influence of a drug. The comparison is made in Table III. In that table the "percentage of uric acid recovered" has in each instance been calculated by ourselves in the manner employed for our own experiments; the figures therefore differ sometimes from those given by the authors, who have not always borne in mind that only four of the five nitrogen atoms of an aminopurine are retained in the uric acid molecule. We have not thought it worth while to take account, in the comparison, of the small proportion of ingested purine that has now and then been recovered in the form of bases. The "case numbers" have been assigned arbitrarily, and merely for the purpose of indicating how many separate individuals have formed the subject of experiments.

The experiments reproduced in Table III have this result in common, that only a fraction of the purine nitrogen ingested appears in the urine as uric acid. This is true even when it can be confidently asserted that the purine nitrogen has been completely absorbed and promptly reexcreted. It has therefore been argued (particularly by Schittenhelm and his coworkers) that uric acid cannot be a terminal product of human metabolism, but that man, admittedly incapable of converting uric acid into allantoin, must transform it into some other simple compound, presumably urea. Now, if the argument be admitted, it follows from Table III that in different subjects, and even in the same subjects at different times, "uricolysis" must take place to very different degrees. The "uricolytic index" for man must range between such widely separate values as 94 (Case 11) and 29 (Cases 6 and 9). Our experience with lower mammals, in which the uricolytic index

TABLE III.

Case No.	Amount administered during experiment.	Purine nitrogen content of preparation.	Duration of experiment.	Theoretical uric acid recovered.	Authority.
	gm.	*gm.*	*days*	*per cent*	
1	20	3.96	1	38	Pollak [10]
2	10	"	1	48	"
3	10	6.58	1	52	Bloch [11]
4	10	"	1	60	"
5	10	"	1	65	"
6	10	"	1	71	"
7	12.21	9.29	1	44	Landau [12]
8	12.34	"	1	47	"
9	10.01	"	1	71	"
10	10.03	"	1	53	"
11	50	6.08	5	6	Frank and Schittenhelm[13]
12	40	"	4	13	"
13	20	"	2	51	"
14	30	6.20	3	29	Brugsch and Schittenhelm[14]
15	30	7.22	3	30	"
16	20	"	2	45	"
17	16	8.0	1	20	Frank and Przedborski[15]
18	15	"	1	37	"
19	16	"	1	13	"
20	16	"	1	18	"
21	3.18	8.68	1	29	Givens and Hunter
	6.05	"	1	14	"
	9.12	"	1	23	"
22	7	"	1	30	"

has almost the character of a constant for the species, makes the existence of such extreme individual variations highly improbable.

[10] Pollak, L., *Deutsch. Arch. f. klin. Med.*, 1907, lxxxviii, 224.

[11] Bloch, B., *ibid.*, 1905, lxxxiii, 499.

[12] Landau, A., *ibid.*, 1909, xcv, 280.

[13] Frank, F., and Schittenhelm, A., *Ztschr. f. physiol. Chem.*, 1909, lxiii, 269.

[14] Brugsch, T., and Schittenhelm, A., *Ztschr. f. exper. Path. u. Therap.*, 1907, iv, 480.

[15] Frank, E., and Przedborski, *Arch. f. exper. Path. u. Pharmakol.*, 1912, lxviii, 349. Strictly speaking, these experiments ought not to appear in the table, for the purine content of the preparation used was assumed, not directly determined, to be "about 8 per cent."

To our minds the failure to recover, after feeding sodium nucleate, more than a very variable percentage of the theoretically possible uric acid finds a much more plausible explanation in some such hypothesis as that of Sivén,[16] namely, that purines entering, or liberated in, the alimentary canal undergo before absorption a varying degree of bacterial destruction. It was along lines suggested by this hypothesis that we had proposed, in the first place, to continue our experiments. Should opportunity permit, we shall return to the problem from this angle.

[16] Sivén, V. O., *Arch. f. d. ges. Physiol.*, 1912, cxlv, 283; 1914, clvii, 582.

THE DETERMINATION OF AMMONIA NITROGEN IN STEER'S URINE.

By DONALD C. COCHRANE.

(From the Institute of Animal Nutrition of the Pennsylvania State College, in cooperation with the Bureau of Animal Industry of the United States Department of Agriculture.)

(Received for publication, September 27, 1915.)

In connection with certain metabolism experiments now in progress in this Institute, the determination of the nitrogen present in the urine of a steer as ammonium compounds and ammonia has become of considerable importance. Recurring peculiarities in the results obtained in some earlier work involving ammonia determinations led to the belief that the chloroform used in the urine as a preservative was inefficient, since in every case urine so treated showed an increase in the ammonia from day to day. Therefore it was planned to test out several preservatives especially as to their ability to prevent the breaking up of nitrogenous substances.

Numerous attempts have been made in this laboratory to differentiate between the nitrogen present in steer's urine as ammonium compounds and that present as ammonia. Braman,[1] however, has shown that practically all the ammonia nitrogen present is in the form of ammonium carbonate with possibly small quantities of ammonia. Simple aeration causes decomposition of ammonium carbonate, and while the addition of sodium chloride prevents a complete breaking up of the carbonate it has been found impossible to obtain a satisfactory separation.

The total quantity of urine excreted by a steer at one voiding was collected, immediately divided into four approximately equal parts, and preserved at about 10°C. during the period covered by analysis. The four samples were treated as follows:

[1] Braman, W. W., *Jour. Biol. Chem.*, 1914, xix, 105.

I No preservative added.
II Chloroform added to saturation.
III Toluene added.
IV Sufficient N sulphuric acid added to render the sample slightly acid, using cochineal as an indicator.

The total nitrogen per cc. of urine was determined in all four samples using the Kjeldahl method. It was necessary, because of the dilution incident to the addition of the sulphuric acid to Sample IV, to compute the results obtained for ammonia nitrogen in terms of the original urine. In doing this, use was made of the total nitrogen in the original urine and the total nitrogen in the acid sample since these determinations were much more accurate than any measurement of volume available.

TABLE I.

Experiment 220, Period I, Steer K, Preliminary.

Ration 7.0 Kg. Clover Hay.

Nitrogen as ammonium compounds expressed as mg. nitrogen per cc. of original urine.

Sample.	1st day.*	2nd day.	3rd day.	4th day.	5th day.	6th day.	7th day.	8th day.	9th day.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I	0.44	0.98	1.93			4.02	4.86	5.74	6.47
II		0.96	1.66			2.43	2.51	2.61	2.64
III		1.39	2.27	2.87		3.44	3.59	3.75	3.77
IV		0.44	0.40	0.44		0.52	0.57	0.59	0.68

* Total nitrogen 8.48 mg. per cc.

Nitrogen as ammonium compounds was determined by Steel's modification of Folin's method.[2] This method was decided upon because of the heavy precipitates of phosphates so often encountered in steer's urine.[3] Duplicate determinations of ammonia nitrogen were made daily using the apparatus described by Folin,[4] except that aeration was obtained through the use of a Crowell blower delivering about 7 liters per minute. Three hours were found to be sufficient for the recovery of the ammonia in all cases.

[2] Steel, M., *Jour. Biol. Chem.*, 1910-11, viii, 365.
[3] Steel, M., and Gies, W. J., *ibid.*, 1908-09, v, 71.
[4] Folin, O., *Ztschr. f. physiol. Chem.*, 1902, xxxvii, 161.

Table I shows the comparative results obtained upon each sample during nine successive days.

An examination of the table reveals rather astonishing results especially in view of the wide use of chloroform as a urinary preservative. The progressive and rapid increase in the ammonia nitrogen in Samples II and III, in which chloroform and toluene were used, would tend to cast doubt on the accuracy of ammonia determinations in the urine of herbivora reported by previous investigators.

The inhibiting action in the case of the sulphuric acid may be ascribed to the bactericidal action of the slight excess of acid[5] over that necessary to neutralize the titratable alkalinity, or possibly to the formation of acid phosphates.[6]

To confirm the conclusions drawn from this experiment a further investigation of the action of sulphuric acid as a retardant of ammoniacal decomposition was undertaken. This covered a ten day period during which the total quantity of urine excreted each day was collected and sampled. Two samples of each daily urine were taken. One sample was treated with N H_2SO_4 as described above; the other was untreated. Both samples were kept in the ice chest until the analyses were made. In every case ammonia determinations were made as soon as possible after the close of the experimental day (6.00 p.m.) and in only two instances was the urine as much as forty hours old at the time of analysis. In the majority of cases fifteen hours, and in one case only two hours elapsed before the determinations were made.

Two composite samples were also made up covering the ten day period. One composite sample was untreated while to the other was added the quantity of sulphuric acid necessary to make the daily aliquot slightly acid.

The acid composite was found to be alkaline at the end of the eleventh day showing that some decomposition had taken place. The quantity of ammonia nitrogen present confirms this, there being twice as much in the acid composite as the average of the acid daily samples.

[5] Endemann, H., *Chem. News*, 1880, xli, 152. Corfield, W. H., and Parks, L. C., Treatment and Utilization of Sewage, London, 3rd edition, 1887.

[6] Rohé, G. H., Textbook of Hygiene, Philadelphia, 2nd edition, 1890, 357. Stutzer, A., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1893, xiv, 116. Kleine, E., Microorganisms and Disease, New York, 2nd edition, 1886, 258.

Table II shows the comparative ammonia nitrogen in the different urines together with that in the corresponding compo sample.

TABLE II.

Experiment 220, Period I, Steer K.

Ration 7.0 Kg. Clover Hay.

Nitrogen in urine as ammonium compounds expressed as mg. nitro per cc. of original urine.

Sample.	1st day.	2nd day.	3rd day.	4th day.	5th day.	6th day.	7th day.	8th day.	9th day.	10th day.	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
Acid.....	0.28	0.25	(0.22*)	0.19	0.19	(0.21§)	0.27	0.34	0.13	(0.19*)	(
Untreated.....	0.48	0.46	(0.53)	0.32	0.32	(0.26)			0.19	(0.42)	(

TABLE III.

Experiment 220, Periods II and III, Steer K.

Rations 2.25 Kg. Clover Hay and 1.5 Kg. Clover Hay with 3.0 Kg. Maize M

Nitrogen in urine as ammonium compounds expressed as mg. nitrogen cc. of original urine.

Sample.	Period II.		Period III.	
	1st day.	Composite.	6th day.	Composit
	mg.	*mg.*	*mg.*	*mg.*
Acid.....	0.21	0.32	(0.57§)	1.95
Untreated....	0.40	1.71	(0.64§)	

* Urine about 40 hours old when determinations were made.

§ Determinations made immediately at close of experimental day.

A comparison of the ammonia nitrogen in the acid with tha the untreated urine furnishes a full confirmation of the res shown in Table I. In every case there is much more nitro present as ammonia in the untreated than in the correspond acid sample.

In order to be sure that sufficient sodium hydroxide was ad to neutralize the slight excess of sulphuric acid and also libe all ammonia, the quantity added was varied with the result Table IV.

A variation from 0.5 to 1.0 gram of NaOH makes no appreciable difference in the quantity of ammonia liberated as shown in Table IV.

TABLE IV.

The Effect of Variations in the Quantity of NaOH on the Ammonia Determinations.

NaOH	Nitrogen.		
	Urine 1514.	Urine 1516.	Urine 1556.
gm.	*mg.*	*mg.*	*mg.*
0.5	1.18	1.08	1.12
0.75	1.13	0.98	
1.0			1.12

An examination of the results obtained on the acid and on the untreated composite sample, Table II, shows the same relative increase in ammonia as was found in the case of the daily urines. That decomposition took place even in the acid composite is shown by comparing it with the average of the acid daily samples from which it was made.

A composite of a series of urines, Table III, Period II, containing a slightly greater quantity of ammonia, treated as above showed an even greater difference between the acid and the untreated sample and about the same increase over the average of the acid daily urines from which it was made.

CONCLUSIONS.

1. Figures for nitrogen as free ammonia in the urine of cattle are unreliable because of the decomposition of ammonium carbonate.

2. Figures for total ammonia nitrogen are worthless unless special precautions are taken to overcome the rapid ammoniacal decomposition.

3. Chloroform and toluene fail to prevent the breaking up of the nitrogenous compounds.

4. Sulphuric acid when added to the urine of a steer in sufficient quantity to fix the ammonia present as carbonate and to slight

Table II shows the comparative ammonia nitrogen in the ten different urines together with that in the corresponding composite sample.

TABLE II.

Experiment 220, Period I, Steer K.

Ration 7.0 Kg. Clover Hay.

Nitrogen in urine as ammonium compounds expressed as mg. nitrogen per cc. of original urine.

Sample.	1st day.	2nd day.	3rd day.	4th day.	5th day.	6th day.	7th day.	8th day.	9th day.	10th day.	Composite.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Acid.....	0.28	0.25	(0.22*)	0.19	0.19	(0.21§)	0.27	0.34	0.13	(0.19*)	0.39
Untreated.....	0.48	0.46	(0.53)	0.32	0.32	(0.26)			0.19	(0.42)	0.77

TABLE III.

Experiment 220, Periods II and III, Steer K.

Rations 2.25 Kg. Clover Hay and 1.5 Kg. Clover Hay with 3.0 Kg. Maize Meal.

Nitrogen in urine as ammonium compounds expressed as mg. nitrogen per cc. of original urine.

Sample.	Period II.		Period III.	
	1st day.	Composite.	6th day.	Composite.
	mg.	*mg.*	*mg.*	*mg.*
Acid.....	0.21	0.32	(0.57§)	1.95
Untreated....	0.40	1.71	(0.64§)	

* Urine about 40 hours old when determinations were made.

§ Determinations made immediately at close of experimental day.

A comparison of the ammonia nitrogen in the acid with that in the untreated urine furnishes a full confirmation of the results shown in Table I. In every case there is much more nitrogen present as ammonia in the untreated than in the corresponding acid sample.

In order to be sure that sufficient sodium hydroxide was added to neutralize the slight excess of sulphuric acid and also liberate all ammonia, the quantity added was varied with the results in Table IV.

A variation from 0.5 to 1.0 gram of NaOH makes no appreciable difference in the quantity of ammonia liberated as shown in Table IV.

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A composite of a series of urines, Table III, Period II, containing a slightly greater quantity of ammonia, treated as above showed an even greater difference between the acid and the untreated sample and about the same increase over the average of the acid daily urines from which it was made.

CONCLUSIONS.

1. Figures for nitrogen as free ammonia in the urine of cattle are unreliable because of the decomposition of ammonium carbonate.

2. Figures for total ammonia nitrogen are worthless unless special precautions are taken to overcome the rapid ammoniacal decomposition.

3. Chloroform and toluene fail to prevent the breaking up of the nitrogenous compounds.

4. Sulphuric acid when added to the urine of a steer in sufficient quantity to fix the ammonia present as carbonate and to slight

excess retards decomposition to such an extent as to allow time for analysis.

5. All ammonia determinations must be made on daily samples of urine because the sulphuric acid does not completely stop decomposition in a composite sample.

STUDIES ON BLOOD FAT.

II. FAT ABSORPTION AND THE BLOOD LIPOIDS.

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(*From the Laboratories of Biological Chemistry of the Harvard Medical School, Boston.*)

(Received for publication, September 25, 1915.)

The mechanism of fat absorption in its relation to the composition of the blood is very little understood and its investigation is necessary as a preliminary step in the study of fat metabolism. The following are some of the problems which present themselves in this connection.

It is known that the fats reach the blood stream in a very fine state of division—the "fat dust" of Munk, the "haemakonien" of Neumann. Is this fine suspension the only provision made to render the fats transportable or is something added to render the suspension more permanent? What is therefore the exact composition of the fat when it reaches the blood stream from the intestine? How does the presence of an excess of foreign fat, such as is present in alimentary lipemia, influence the relations of the normal lipoids of the blood?

The present views in this regard are that the fats, after their hydrolysis in the intestine, are resynthesized during their passage through the intestinal wall and pass into the blood stream in essentially the form in which they were ingested, *i.e.*, as glycerides, and that alimentary lipemia is due to nothing more than the addition of these glycerides. There is, however, a growing feeling that the process is not so simple. On the one hand, examination of the blood in many pathological conditions has shown that the lipemia found is frequently a "lipoidemia,"[1] in that along with the increase of true fat there is also an increase of lecithin and cholesterol; and on the other hand a school of French investi-

[1] Müller, J., *Ztschr. f. physiol. Chem.*, 1913, lxxxvi, 469.

gators[2] have found that there is normally a fairly constant relationship between the lipoid constituents of various tissues including the red blood corpuscles and that therefore a similar constancy of relationship might be expected in the lipoids of the blood serum.

Some evidence has already been presented to show that the fats do not reach the blood stream in exactly the form in which they were taken in as food. It was found,[3] for example, that the fat of the chyle had a somewhat different composition from that of the food fat, as shown by a different iodine number and melting point, and that the direction and extent of the changes depended on the nature of the fat fed. With the harder fats the melting point was lowered and the iodine number raised, while with the liquid fats the reverse was the case. These changes were much greater than could be accounted for by the fatty material of fasting chyle. Whether the differences were due to admixture of material supplied by the blood during absorption (for the purpose of rendering transport easier?), or whether they were due to changes in the fats themselves during the process, was not clear. As regards changes in the blood lipoids during fat absorption we have very little evidence. Reicher[4] in three experiments on dogs found great increases in lecithin (82 per cent) and cholesterol (65 per cent) during fat absorption, while at the same time the average fat increase was 53 per cent. Terroine[5] found a parallelism between the increase of fat and of cholesterol in the blood during fat absorption. Greenwald[6] states that the lipoid phosphorus of the blood serum does not appear to increase during fat absorption. The possibility of such changes in the blood lipoids during fat absorption is of interest not only from the point of view discussed above but also because of their probable influence on various blood reactions, such as hemolysis, coagulation, immunity, etc., all of which have been claimed to be dependent upon or be influenced by the lipoids. For the same

[2] Mayer, A., and Schaeffer, G., *Jour. de physiol. et de path. gén.*, 1913, **xv**, 984. Terroine, E. F., *ibid.*, 1914, xvi, 212.

[3] Bloor, W. R., *Jour. Biol. Chem.*, 1913-14, xvi, 517.

[4] Reicher, K., *Verhandl. d. Cong. f. inn. Med.*, 1911, xxviii, 327.

[5] Terroine, *Jour. de physiol. et de path. gén.*, 1914, xvi, 386.

[6] Greenwald, I., *Jour. Biol. Chem.*, 1915, xxi, 29.

reason such increases would be of interest clinically from their influence on the various blood tests used in diagnosis. It seemed desirable, therefore, to undertake a study of the effect of fat absorption on the blood lipoids and the following experiments were carried out for that purpose.

EXPERIMENTAL.

Dogs which had received no food for about twenty-four hours were given a feeding of fat and then determinations of the blood lipoids were made on blood samples taken at intervals over a period of about eight hours, the first sample being taken just before the feeding, the others at intervals of one or more hours. The blood was analyzed for (a) total fat (fatty acids plus cholesterol), (b) cholesterol, and (c) "lecithin" according to the following scheme.

Preparation of the Sample.

3 cc. of blood were drawn from the jugular vein, by means of a needle and a short length of rubber tubing, into a pipette containing a little powdered oxalate and run slowly (a slow stream of drops) into about 75 cc. of a mixture of alcohol and ether (three parts 95 per cent alcohol and ether, both freshly distilled) in a 100 cc. graduated flask, the liquid in the flask being kept in motion during the emptying of the pipette. The contents of the flask were then raised just to boiling by immersion of the flask in a boiling water bath with shaking to prevent superheating, then cooled under the tap to room temperature, made up to the mark with more alcohol-ether, mixed, and filtered. The filtrates were clear and practically colorless and if well stoppered and put in a cool dark place could be kept unchanged for several months.

Total Fat (Fatty Acids plus Cholesterol).

The method used was described in detail in a previous article.[7] It is a method depending on the precipitation of the fat in water under suitable conditions and determination of its amount by the use of the nephelometer. The procedure is briefly as follows.

10 cc. of the blood extract, containing about 2 mg. of fat, are measured into a small beaker and saponified by adding 2 cc. of N sodium ethylate and evaporating off the alcohol. (It should not be allowed to come to

[7] Bloor, *Jour. Biol. Chem.*, 1911, xvii, 377.

complete dryness or the results will occasionally be too high.) 5 cc. of the alcohol-ether mixture are run in and the solution is raised just to boiling, then 50 cc. of distilled water are added. (The original directions call for 100 cc. of water but it has been found that more satisfactory readings can be made with the denser suspension as above.)

A standard solution is prepared by measuring 5 cc. of an alcohol-ether solution of oleic acid, containing about 2 mg. of oleic acid, with stirring, into 50 cc. of water in a similar small beaker. To the standard and test solutions are then added simultaneously from pipettes and with stirring 10 cc. of dilute HCl (1 part concentrated HCl with 3 parts of water) and the mixtures allowed to stand for five minutes, after which the nephelometer tubes are filled and readings made as usual. Special care is required to avoid the presence of bubbles which are very liable to form on the sides of the tubes owing to the presence of ether in the liquid. After completion of the reading the tubes should be examined and if bubbles are present they should be removed by careful inversion of the tubes and the readings repeated. The presence of bubbles increases the apparent value of the solution. The values of the solutions are inversely proportional to the readings and calculations are made as in colorimetric work.

The nephelometer used was of the Richards type made by a modification of the Duboscq colorimeter.[8]

Cholesterol.

The Autenrieth-Funk method[9] was applied to the blood extract as follows.

10 cc. of the extract (containing about 0.5 mg. of cholesterol) were measured with a pipette into a small beaker, 2 cc. of sodium ethylate added, and the whole was evaporated to dryness and dried half an hour. 5 cc. of a standard cholesterol solution, containing 0.5 mg. of cholesterol and about 2 mg. of oleic acid were measured into a beaker and similarly treated. The cholesterol was extracted from both by boiling out with 5 cc. portions of dry chloroform for three periods of five minutes each, decanting the extracts through a small filter into another beaker, and after evaporating the extracts to small bulk, transferring to 10 cc. graduates and making up to 6 cc. From this point on the treatment was the same as in the Autenrieth-Funk procedure. 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulphuric acid were added, the solutions mixed by inverting the graduates, then set away in the dark at 30–32° C. for fifteen minutes, after which comparisons were made in the colorimeter. The Duboscq colorimeter was used, the glass cups being set in plaster of Paris since the ordinary setting material is soluble in chloroform. The white glass plate was used in place of the mirror.

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[8] Bloor, *Jour. Biol. Chem.*, 1915, xxii, 145.

[9] Autenrieth, W., and Funk, A., *München. med. Wchnschr.*, 1913, lx, 1243.

As has been pointed out by Klein and Dinkin,[10] the chloroform extract of the cholesterol in the Autenrieth-Funk method contains some brownish color which makes comparison with the standard color difficult and more or less uncertain. The saponification and drying of both standard and test as above was done to overcome the effect of the brownish tint by producing it in both the standard and test solutions. The device overcomes the difficulty to a considerable extent and makes satisfactory readings possible in all but a few cases.

Method for Lecithin.

The method used was described in a previous article.[11] It depends on the precipitation of the phosphoric acid of the lecithin, after ashing, with silver nitrate under suitable conditions and the determination of the amount of the precipitate by the use of the nephelometer. The procedure is as follows.

10 cc. of the blood extract (containing about 1.2 mg. of "lecithin" or 0.15 mg. H_3PO_4) are measured into a 200 x 25 mm. Jena test-tube, three or four glass beads of 3 mm. diameter are added, and the liquid is evaporated to dryness by immersion in a boiling water bath. The tube is shaken frequently until boiling has actively begun, after which the evaporation proceeds quietly to dryness. The contents of the tubes are left in the bath for a short time (about fifteen minutes) after the material comes to dryness to ensure the removal of the last traces of alcohol, which would interfere with the oxidation. To the contents of the tube are added 1.5 cc. of equal parts of concentrated sulphuric and nitric acids and the whole is heated over a micro burner, at first gently with a very low flame for at least five minutes, then with increasing heat until the red fumes are driven off, and finally the sulphuric acid is boiled for about ten minutes.

The tube is cooled slightly, two drops of a 0.25 per cent cane-sugar solution are added, and then the solution is boiled for another minute, after which it is cooled and the sides are rinsed down with about 3 cc. of water. The object of the treatment with the cane-sugar is to break up a partial combination of the phosphoric acid with the nitric acid formed during the digestion which does not precipitate with the silver reagent. The slight charring produced by the cane-sugar should quickly disappear on boiling. If it does not it must be removed by the addition of a drop of nitric acid and further boiling.

After the above treatment the solution in the tube is neutralized, and then rendered faintly alkaline to phenolphthalein, the process being conveniently accomplished as follows: One drop of 0.3 per cent phenolphthalein solution is added, then 20 per cent NaOH (free from chlorides) is run

[10] Klein, W., and Dinkin, L., *Ztschr. f. physiol. Chem.*, 1914, xcii, 302.

[11] Bloor, *Jour. Biol. Chem.*, 1915, xxii, 133.

in to alkalinity, noting the amount added. The solution is brought back to acidity with $\frac{N}{2}$ H_2SO_4, then, after cooling to room temperature, rendered just alkaline with $\frac{N}{10}$ NaOH. 1 cc. of 10 per cent $(NH_4)_2SO_4$ and 1.5 cc. $\frac{N}{10}$ NaOH are added and the solution is made up to 10 cc. (indicated with sufficient accuracy by a scratch on the tube). A standard phosphate solution is similarly prepared as follows: 3 cc. of a solution of acid potassium phosphate (containing 0.15 mg. of H_3PO_4) is measured into a similar Jena test-tube; one drop of phenolphthalein is added, and then the amount of 20 per cent NaOH that was required to neutralize the sulphuric acid of the test solution is run in. Concentrated sulphuric acid is added to neutralization, the excess of sulphuric acid removed by a drop or two of the strong alkali, the solution cooled, then neutralized as above. 1 cc. of 10 per cent $(NH_4)_2SO_4$ and 1.5 cc. $\frac{N}{10}$ NaOH are added and the solution is made up to the 10 cc. mark on the tube. Two samples of 10 cc. each of 1.5 per cent neutral $AgNO_3$ are measured into 25 cc. glass-stoppered graduated flasks and the standard and test solutions in the test-tubes are added through a funnel with the stem drawn out so that the 10 cc. are delivered in about fifteen seconds. The liquid in the flasks is gently rotated while the phosphate solution is being run in, after which the test-tubes are rinsed out with small amounts of distilled water, and the rinse water is run in through the funnel. Finally the liquid in the flask is brought up to the mark by rinsing the funnel with distilled water, the whole well mixed, and readings are made in the nephelometer. Chlorides must of course be rigidly excluded especially after the acid digestion—accomplished by using chlorine-free reagents and by the liberal use of good distilled water. For the comparison the two nephelometer tubes after being rinsed with the solutions are filled to the same height and placed in the nephelometer with the standard tube always on the same side. The movable jacket on the standard tube is set at a convenient point and comparisons are made in the usual way.

The experiments were as follows.

Experiment I.—Dog 10, normal, weight 5 kg. Fed 30 cc. of olive oil and about 50 gm. of lean meat at 9.20 a.m. Blood samples taken before and at 7 and 9 hours after feeding.

Experiment II.—Dog 14, normal, weight 11 kg. Fed 125 gm. of lean meat and 100 cc. of olive oil. Blood samples taken before and at 6 and 8 hours after feeding. A small amount of oil was vomited.

Experiment III.—Dog 21, very thin, weight 7 kg. Fed 50 cc. of olive oil and 50 cc. of water. Blood samples taken before and at hourly intervals for 7 hours.

Experiment IV.—Dog 21, condition about the same as in Experiment III. Fed 50 gm. of butter. Blood samples before and at hourly intervals for 7 hours.

Experiment V.—Dog 21. Fed 1 pint of cream (15 per cent or 65 gm. of fat). Blood samples taken before and hourly for 8 hours.

Experiment VI.—Dog 21. The animal had now become fat. Fed 50 cc. olive oil with bread. Blood samples taken before and at hourly inters for 5 hours after.

Experiment VII.—Dog 23, old female, normal, weight 6 kg. Refused olive oil and 45 cc. were given by tube. The animal was uncomforle for about 2 hours, vomiting a small amount of the oil, but after that aved normally. Blood samples taken before and hourly for 7 hours er.

Experiment VIII.—Dog 23. Fed 100 gm. of lean meat, 50 gm. of beef t, and 30 gm. of butter. Blood samples before and hourly for 8 hours er.

Experiment IX.—Dog 23. Fed 100 gm. of butter and 25 gm. of lean meat. od samples before and at two hourly periods for 8 hours. This time, n Experiment VII, the fat did not seem to agree with the dog. It nited much of the fat at the end of the first hour but ate it again. nited again at the second hour and again at the fifth, losing in all probly half the fat.

Experiment X.—Dog 24, normal female, weight 8.7 kg., in good conon. Fed 125 gm. of butter and 25 gm. of lean meat. Blood samples ore and at hourly intervals for 8 hours.

Experiment XI.—Dog 24, this time in poor condition. Fed 75 cc. of e oil and 25 gm. of meat. Blood samples before and at hourly inters for 8 hours.

The results of the experiments are given in the table.

SUMMARY AND DISCUSSION.

The fatty acids show the ordinary increase of alimentary emia but the extent of increase varies considerably in different mals and in the same animal at different periods even when the ount and kind of fat are the same. Similar variations have en reported by Terroine[12] and by Mendel and Baumann[13] and ther study of the conditions controlling this phenomenon is irable. The variations in cholesterol are small and irregular l in several of the experiments there is no appreciable change oughout the period of observation. Cholesterol, therefore, pears to take at most a minor part in the phenomena of fat sorption.

Lecithin is found to increase in all the experiments, the inases varying from 10 to 35 per cent with an average of about 20

[12] Terroine, *loc. cit.*

[13] Mendel, L. B., and Baumann, E. J., *Jour. Biol. Chem.*, 1915, xxii, 165.

Variations in the Blood Lipoi

Time.	Experiment I.			Experiment II.			Experiment III.			Experiment IV.			Experiment V		
	F.A.*	C.	L.	F.A.	C.	L.	F.A.	C.	L.	F.A.	C.	L.	F.A.	C.	L
Before	0.60	0.17	0.31	0.60	0.14	0.34				0.43	0.17	0.32	0.54	0.22	0.1
hrs.															
1							0.54	0.17	0.38	0.43	0.17	0.31	0.54	0.22	0.3
2							0.61	0.18	0.39	0.51	0.17	0.35	0.54	0.21	0.4
3							0.67	0.16	0.42	0.52	0.16	0.39	0.58	0.22	0.4
4							0.69	0.16	0.41	0.51	0.17	0.39	0.81	0.19	0.4
5							0.77	0.16	0.42	0.58	0.18	0.40	0.77	0.20	0.4
6				1.20	0.14	0.37	0.70	0.15	0.40	0.51	0.17	0.39	0.78	0.20	0.4
7	1.30	0.17	0.42				0.65	0.16	0.42	0.50	0.16	0.40	0.64	0.21	0.4
8				0.90	0.16	0.38							0.61	0.19	0.4
9	0.90	0.17	0.41												

* F. A. Fatty acids ("total fat" minus cholesterol).
C. Cholesterol.
L. Lecithin ($H_3PO_4 \times 8$).
The figures are in per cent of the whole blood.

per cent. In a general way the increases are parallel to those of the fatty acids but there is no exact parallelism and the two are often markedly different, the lecithin increase frequently coming later, as in Experiment XI, where the lecithin does not begin to increase until after the maximum of fat increase has passed. In many cases there is no definite maximum in the lecithin values, the higher values continuing for several hours; also these high values generally persist after the fatty acid maximum has passed. Anything more than a general relationship between the lecithin and fatty acid values is perhaps hardly to be expected because of the numerous factors controlling the entry into and the departure from the blood of these two constituents.

The increase of lecithin in the blood during fat absorption is interesting in view of the growing acceptance of Leathes'[14] hypothesis that lecithin is a stage through which the fats must pass before they can be utilized in metabolism. It is possible that the extra lecithin may represent that part of the absorbed fat which is intended for immediate use and which has been synthesized for that purpose from the material absorbed from the intestine.

[14] Leathes, J. B., The Fats, London, 1913, 115.

Fat Absorption.

ent VI.		Experiment VII.			Experiment VIII.			Experiment IX.			Experiment X.			Experiment XI.		
C.	L.	F.A.	C.	L.	F.A.	C.	L.	F.A.	C.	L.	F.A.	C.	L.	F.A.	C.	L.
.24	0.37	0.50	0.18	0.34	0.57	0.15	0.32	0.52	0.13	0.32	0.48	0.17	0.36	0.68	0.17	0.36
.25	0.38	0.55	0.17	0.35	0.56	0.16	0.32				0.51	0.17	0.37	0.76	0.17	0.35
.20	0.40	0.66	0.16	0.36	0.60	0.16	0.33	0.56	0.13	0.32	0.82	0.17	0.42	1.01	0.18	0.36
.22	0.43	0.72	0.15	0.37	0.81	0.16	0.33				0.94	0.17	0.46	1.09	0.18	0.36
.24	0.39	0.58	0.14	0.35	0.87	0.15	0.36	0.66	0.13	0.37	1.02	0.17	0.45	1.44	0.17	0.36
.24	0.37	0.54	0.14	0.37	0.78	0.15	0.36					0.17	0.44	1.27	0.17	0.40
		0.53	0.15	0.35	0.78	0.15	0.39	0.58	0.13	0.38	0.92	0.18	0.45	0.85	0.17	0.42
		0.51	0.14	0.35	0.70	0.15	0.38				0.82	0.18	0.44	0.84	0.18	0.41
					0.70	0.14	0.36	0.55	0.13	0.41	0.72	0.18	0.42	0.80	0.17	0.39

ccording to Leathes' hypothesis also, the synthesis would take ace in the liver; and the old observations of Munk[15] that there an accumulation of fat droplets in the liver during fat absorpn and the later one of Leathes[16] that the fat of the liver ineases during this process give support to the probability that the er has some function in fat absorption other than the secretion of le. An objection to the assignment of this function to the liver the definite anatomical arrangement whereby apparently the sorbed fat is made to avoid the liver. It passes by way of the oracic duct directly into the general circulation. However, ly 60 to 70 per cent of the absorbed fat can be accounted for in e thoracic duct and it is quite possible that the remaining 30 40 per cent is absorbed by the intestinal capillaries and passes rectly to the liver by way of the portal circulation, thus providg abundant material for the formation of the lecithin. The servations of d'Errico[17] that, during fat absorption, the fat ntent of the portal blood is always higher than that of the jugur, bear out this assumption.

On the other hand the possibility cannot be excluded that the testine may synthesize the lecithin just as it does the fats.

[15] Munk, I., *Ergebn. d. Physiol.*, 1902, i, 322.

[16] Leathes, J. B., and Meyer-Wedell, L., *Jour. Physiol.*, 1909, xxxviii, . xxxviii–xl.

[17] d'Errico, G., *Arch. di fisiol.*, 1907, iv, 513.

Analysis of the chyle for its lecithin content during fat absorption would probably give important information on this point but such analyses are not at present available. However if fats and lecithin were formed simultaneously in this way, a closer parallelism between the two in the blood would be expected and the fact that very frequently the increases in lecithin come later and generally persist longer than those of the fats indicates rather that the increase in fat in the blood is the stimulus which starts the lecithin formation and that the intestine is not the seat of formation. Such an explanation would also account for the "[redacted]poidemia" mentioned above. Increased fat in the blood, from whatever source, would result in increased lecithin production.

THE CONFIGURATION OF SOME OF THE HIGHER MONOSACCHARIDES.

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In 1890 Emil Fischer[2] prepared from *d*-mannose a heptose, octose, and nonose. He later prepared from *d*-glucose,[3] α- and β-gluco-heptose,[4] a glucooctose and a glucononose, and from *d*-galactose,[5] α- and β-galaheptose and a galaoctose. Of the ten new sugars only the mannononose fermented with yeast. The configuration of mannose was not then known, so naturally no attempt was made to obtain the configuration of the nonose. When it was found later that the glucononose was not fermentable, the configurations of the two nonoses became of great interest but it was not found practicable at that time to establish them.

In the present paper the configuration of the α- and β-galaheptose, the α- and β-mannoheptose and the α-α- and β-α-mannooctose derivatives is obtained. This leaves the configuration of one of the carbon atoms of the fermentable mannononose still unknown. To ascertain this will be a matter of some little time.

The two heptites derived from *d*-mannose have the configura-

[1] A part of this work was done in the Laboratory of Pharmacology of the University of Wisconsin, and was reported at the 1913 meeting of the Society of Biological Chemists.

[2] Fischer, E., and Passmore, F., *Ber. d. deutsch. chem. Gesellsch.*, 1890, xxiii, 2226.

[3] Fischer, E., *Ann. d. Chem.*, 1892, cclxx, 64. Phillipe, L. H., *Ann. de chim. et de phys.*, 1912, xxvi, 289, has also prepared a glucodecose and confirmed and slightly elaborated Fischer's other results with the higher glucoses.

[4] The designations α and β are used merely to distinguish the two series of products formed in each cyanhydrin synthesis.

[5] Fischer, *Ann. d. Chem.*, 1895, cclxxxviii, 139.

tions represented by Formulæ I and II, and the two *d*-galaheptites those represented by Formulæ III and IV.

CH_2OH	CH_2OH	CH_2OH	CH_2OH
HCOH	HOCH	HCOH	HOCH
HOCH	HOCH	HCOH	HCOH
HOCH	HOCH	HOCH	HOCH
HCOH	HCOH	HOCH	HOCH
HCOH	HCOH	HCOH	HCOH
CH_2OH	CH_2OH	CH_2OH	CH_2OH
I	II	III	IV

Formulæ I and III are optical antipodes as can be seen by reference to the models or by rotating one of the projections 180° in the plane of the paper.

The properties of *d*-α-mannoheptite and *d*-α-galaheptite are known.

	d-α-Mannoheptite[8]	*d*-α-Galaheptite[7]
M. p. (corrected)	188°	187–188°
$[\alpha]_D^{20}$ (in saturated borax solution)	+4.9° (approximately)	−4.35°

d-α-Mannoheptite and *l*-α-mannoheptite unite to form a racemic compound[8] melting at 203° (corrected). I found that *d*-α-mannoheptite and *l*-α-galaheptite combine to form a compound melting at 205° (corrected). The two active components consist of fine needles in both cases; the two racemic compounds are tablet-like crystals. From these facts it seems beyond question that *d*-α-mannoheptite is the antipode of *d*-α-galaheptite. Formulæ I and III must therefore be assigned to the α-heptites, leaving II and IV for the β-compounds.

Crystallized *d*-α-mannoheptaric[9] acid was also prepared for the first time and found to have the same melting point as *d*-α-galaheptaric acid but the opposite rotation. The two acids are thus antipodes, as was to have been expected.

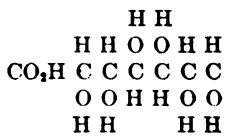
[6] Fischer and Passmore, *loc. cit.*, 2232.

[7] Fischer, *Ann. d. Chem.*, 1895, cclxxxviii, 147.

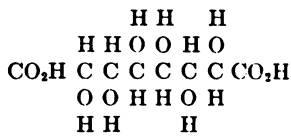
[8] Smith, W. S., *ibid.*, 1892-93, cclxxii, 189.

[9] The termination "-aric" is proposed for the dibasic acids formed on oxidation of the monoses. Thus *d*-saccharic acid becomes *d*-glucohexaric acid, mucic acid becomes galahexaric acid, the pentahydroxypimelic acids become heptaric acids, etc.

On oxidizing *d*-α-α-mannooctonic acid lactone with nitric acid a double lactone of the octaric acid crystallized out. It was so insoluble that it could not be polarized directly but on dissolving it in an excess of NaOH and neutralizing, the solution was found to be absolutely inactive. The two possible configurations for the dibasic acid are these:



V



VI

V is inactive and therefore represents the α-α-mannooctaric acid, while VI being active represents the as yet unknown β-α-compound. Since there is a slight chance that the active acid will have a very slight rotation it will be advisable to wait till the β-α-acid is prepared before accepting this configuration unreservedly. The Na salt of the α-α-acid certainly has a specific rotation of less than 1° and probably less than 0.3°, which makes the chance of its being active very slight indeed.

Owing to lack of material, Fischer's statements in regard to the mannononose could not be confirmed, but no facts were found at variance with his *Berichte* article.[10]

By the action of hydrocyanic acid on mannose Fischer obtained a mannoheptonic acid in 87 per cent yield with great regularity.[11] He used a syrupy mannose,[12] while in most of this work the crystallized sugar was used and the synthesis carried out at 40° instead of 18°. Under these conditions 60 to 80 per cent of the above acid is obtained, while 5–6 per cent of a second acid (which

[10] Fischer says in his collected papers on the carbohydrates (p. 582) that new observations are needed to explain certain differences that he found. It is possible that he obtained the α-acid in one synthesis and the β-acid in another.

[11] Fischer, E., and Hirschberger, J., *Ber. d. deutsch. chem. Gesellsch.*, 1889, xxii, 370. Smith, *Ann. d. Chem.*, 1892-93, cclxxii, 182. Hartmann, G., *ibid.*, 1892-93, cclxxii, 190.

[12] Crystallized *d*-mannose was not obtained until 1896 by van Ekenstein. Compare B. Tollens in Abderhalden's *Handb. der biochem. Arbeitsmethoden*, 1909–10, ii, 74.

I have called the β-acid) can be isolated by means of phenylhydrazine. The same acid can also be obtained by heating the α-acid with pyridine. There seems to be no reason for doubting that it is the β-acid. Fischer's failure to find it can be explained either by the fact that practically none of it was formed in his syntheses or by the fact that it probably cannot be isolated when an impure mannose is used. The phenylhydrazide of the β-acid is the only crystalline compound that could be obtained.

Finally both *d*-β-mannoheptite and *d*-β-galaheptite were prepared. The first melted at 151°, the second at 141–144°, and their solubilities are different. They cannot be antipodes of each other, nor can *d*-β-mannoheptite be the antipode of *d*-α-galaheptite or *d*-β-galaheptite of *d*-α-mannoheptite.[13]

Preparation of α- and β-Mannoheptonic Acid from Mannose.[14]

100 grams crystallized *d*-mannose are dissolved in 500 cc. water in a glass-stoppered bottle, 135 cc. 12 per cent HCN and 0.5–1 cc. concentrated ammonia are added, and the mixture is warmed to about 35°. The solution then generally warms up spontaneously and should be kept at about 40°. After ½ to 2 hours some amide usually separates out. After 24 to 48 hours the solution is boiled with 2 to 4 liters of water[15] and 160 grams of crystallized barium hydroxide until no more ammonia is given off. This takes from 3 to 6 hours and water must be added from time to time to replace that lost by evaporation. The excess of barium is precipitated with CO_2, water added if necessary, and the boiling solution filtered by suction. After decolorization with animal charcoal the solution is evaporated, at first over a free flame, until crystallization begins. On cooling, the barium salt of the α-acid is deposited in indistinctly crystalline spheres. The salt is filtered off by suction and washed with water. A second crop of crystals can be obtained, but it is not advisable to carry the evaporation too far. The barium in the second mother liquor

[13] Further experimental details will be published on these two heptites and on *d*-β-mannoheptose later.

[14] Compare Fischer and Hirschberger, *loc. cit.*

[15] Cheap enamelled kettles which can be discarded when eaten through have been found very convenient for this.

s precipitated with exactly the necessary amount of sulphuric cid, the barium sulphate filtered off, and the diluted filtrate oiled with cadmium carbonate and cadmium hydroxide till neural. (CO_2 can be passed in if the solution becomes alkaline.) fter filtration the α-cadmium heptonate is separated as completely as possible by crystallization and the cadmium removed rom the final filtrate with H_2S. The solution is heated in the ater bath with an excess of phenylhydrazine for two hours and vaporated on the water bath till the residue is nearly dry. Use f an electric fan at first and frequent stirring at the end ensures slightly purer product. The residue is rubbed up several times ith ether to remove the excess of phenylhydrazine. After reoval of the ether by gentle heating, the mass is placed in a conial beaker, covered with absolute alcohol, and boiled to dissolve ny formylhydrazide present. After cooling, the alcohol is filtered ff and the product, which now consists of nearly pure β-phenylydrazide, crystallized out of ten to fifteen parts of 70 per cent lcohol. The yield of α-acid varied from 60 to 80 per cent, the ield of β-acid was about 5 per cent. The air dried substance ontained no water of crystallization.

d-β-Mannoheptonic Acid Phenylhydrazide.

0.2302 gm. of substance gave 0.4184 gm. CO_2 and 0.1389 gm. H_2O.

	Calculated for $C_{13}H_{20}N_2O_7$ (316.16):	Found:
C	49.34	49.57
H	6.38	6.75

The substance crystallizes out of 70 per cent alcohol in rosettes f colorless needles. It is soluble in about twelve parts of cold ater and fifteen parts of boiling 70 per cent alcohol, insoluble in bsolute alcohol, ether, and acetone. It melts at 190° (uncorected).

Rotation.—The crude product was twice recrystallized out of 70 per ent alcohol and dissolved in warm water.

$$[\alpha]_D^{27} = \frac{-2.26° \times 32.193}{2 \times 1.3888 \times 1.014} = -25.8°$$

The acid was prepared in the usual way from the phenylhydrazide. Neither it nor the lactone could be obtained in the crystalline form. It is easily soluble in water, difficultly soluble in absolute alcohol, insoluble in ether.

Preparation of the β-Acid from the α-Acid by Heating with Pyridine.

5 grams *d*-α-mannoheptonic acid lactone, 25 cc. water, and 5 cc. pyridine were heated in a sealed tube for four hours at 137–142°. Slight browning occurred. The pyridine was driven off by boiling with 5 grams of barium hydroxide and the two acids were separated by the process detailed above. There was thus obtained about 50 per cent of the unchanged α-acid and 10 to 15 per cent of the β-acid. The β-phenylhydrazide melted at 190° and resembled in appearance, crystal form, and solubility the product obtained directly from mannose. On mixing the two products there was no depression of the melting point.

Analysis.—The air dried substance lost 0.5 per cent on drying in vacuum at 100° over P_2O_5. 0.1810 gm. of substance gave 0.3269 gm. CO_2 and 0.1081 gm. H_2O. 0.1538 gm. of substance gave 13.0 cc. N_2 at 744 mm. and 25° over 33 per cent KOH.

	Calculated for $C_{13}H_{20}N_2O_7$:	Found:
C	49.34	49.26
H	6.38	6.68
N	8.86	9.25

The β-acid can also be converted into the α-acid. 5 grams of pure *d*-β-phenylhydrazide were converted into the free acid and the solution was heated with 5 cc. of pyridine for three hours at 137–142° in an autoclave. 5 grams of barium hydroxide were added and the pyridine was expelled by boiling. CO_2 was passed in till the solution was neutral. The boiling solution was filtered and concentrated. 1.7 grams of the barium salt of α-mannoheptonic acid crystallized out. This was identified by its rather characteristic crystal form and by the crystal form and melting point of the phenylhydrazide.

Preparation of d-β-Mannoheptose.

The free *d*-β-mannoheptonic acid was heated in a vacuum for several hours at 100° in order to convert it as fully as possible into the lactone. It was then dissolved in ten parts of water, cooled to freezing, and reduced by shaking with 2.5 per cent sodium amalgam, adding H_2SO_4 every minute or so as the reaction approached the neutral point. The amalgam was added in three lots, four to five times the weight of lactone being added each time, and the reaction was stopped when hydrogen began to be given off freely. The solution then reduced about nine times its volume of mixed Fehling's. The mercury was filtered off and an excess of sodium hydroxide added. After one-half hour the solution was neutralized with sulphuric acid and evaporated till crystallization began. It was then poured into twelve volumes of boiling 95 per cent alcohol. After cooling, the salts were filtered off, dried, dissolved in water, and reprecipitated. The alcoholic solutions were united and evaporated to a syrup. It could not be crystallized. No crystallized phenylhydrazone or parabromphenylhydrazone could be obtained. The osazone was formed by heating with sodium acetate and an excess of phenylhydrazine. It was recrystallized out of absolute alcohol and melted at 210°. A specimen of *d*-α-mannoheptosazone melted at 205°. No further attempt was made to identify the osazone.

Paranitrophenylhydrazone of d-β-Mannoheptose.

The sugar content of the syrup was roughly estimated by reduction of Fehling's and it was then boiled with an equal weight of paranitrophenylhydrazine in ten parts of 50 per cent alcohol under a reflux. At the end of one hour a great deal of paranitrophenylhydrazine was still present so the boiling was continued for two hours and the solution allowed to stand thirty-six hours. The product crystallized out slowly in structureless balls. Yield: about 130 per cent of the sugar. It was boiled out with four parts of benzene and crystallized out of water.

For analysis it was recrystallized out of water till only traces of paranitrophenylhydrazine were left in the mother liquor.

The substance dried over night over H_2SO_4 lost only a trace of water on heating to 76° in vacuum over P_2O_5.

0.1582 gm. of substance gave 0.2615 gm. CO_2 and 0.0878 gm. H_2O.
0.1739 gm. of substance gave 19.8 cc. N_2 at 27° and 759 mm. over 33 per cent KOH.

	Calculated for $C_{13}H_{19}N_3O_8$ (345.15):	Found:
C	45.20	45.08
H	5.55	6.21
N	12.18	12.58

The substance softens at 190°, melts at 198°, and decomposes at 203°. It crystallizes out of water in rosettes of yellow or orange needles. It can also be crystallized out of 95 per cent alcohol but is almost insoluble in ether and benzene.

d-β-Mannoheptose.

The paranitrophenylhydrazone was boiled for fifteen minutes in fifteen times its weight of water with an excess of benzaldehyde. It was cooled, filtered, extracted with ether three or four times, boiled with a little charcoal, and evaporated to a syrup. The sugar crystallized out on cooling and rubbing with a little alcohol. The yield was about 70 per cent of the theory, in reality probably nearly quantitative. The sugar can be recrystallized out of about half its weight of water but the mother liquor is very syrupy and as it was desired to obtain all the heptite possible the heptose was not purified. It is easily soluble in 95 per cent alcohol and moderately soluble even in cold absolute alcohol but no concentration of alcohol could be found from which it would crystallize satisfactorily.

d-β-Mannoheptite.

3 grams of crude heptose were reduced by shaking with sodium amalgam in 10 per cent solution. The solution was neutralized three times an hour with sulphuric acid. 160 grams of amalgam and 20 cc. 5 N H_2SO_4 were used and the reduction was complete in five hours. The solution was neutralized, filtered, evaporated to about 40 cc. and poured into 500 cc. of 95 per cent alcohol. The sodium sulphate was filtered off, dried, dissolved in water, and reprecipitated. The alcoholic solutions were united and evapo-

rated to a syrup. The heptite crystallized easily in rosettes of needles. The entire quantity was taken up in 95 per cent alcohol, filtered hot, and evaporated to a small volume. 1.7 grams crystallized out. 0.5 gram was obtained from the mother liquor. Both lots were united and recrystallized out of 80 per cent alcohol and then out of one part of water. This was used for polarization. The heptite crystallizes in thick needles and rosettes of needles out of 80 per cent alcohol and water. The air dried substance does not lose weight when heated to 76° in vacuum over P_2O_5. It softens at 150–153° and melts to a clear liquid at 217° (uncorrected).

Analysis.—The sample analyzed contained no water of crystallization but contained 0.5 per cent ash. 0.1427 gm. ash free substance gave 0.2049 gm. CO_2 and 0.0998 gm. H_2O.

	Calculated for $C_7H_{16}O_7$ (212.13):	Found
C	39.60	39.20
H	7.64	7.83

$$[\alpha]_D^{22} = \frac{+ 0.235 \times 8.4833}{1.037 \times 0.8462} = + 2.27°$$

d-β-Galaheptite.

Crude *d*-β-galaheptose[16] was reduced in 10 per cent solution with 2.5 per cent sodium amalgam. The solution was kept acid at first so that long shaking and much amalgam were necessary. If reduced in the usual way in slightly alkaline solution probably fifty times its weight of amalgam and 8 to 12 hours' shaking would have been sufficient. The solution was filtered from the mercury, evaporated to crystallization, and poured into ten volumes of 95 per cent alcohol. The salts were filtered off, dried, dissolved in water, and the precipitation was repeated. The alcoholic solutions were united and evaporated to a syrup which crystallized on covering with absolute alcohol and rubbing. It was dissolved in a small quantity of water and hot alcohol added till cloudy. On cooling it crystallized out in rosettes of needles. This was repeated and the substance then recrystallized twice out of 70 per cent alcohol. Yield: about 30 per cent.

[16] Fischer, *Ann. d. Chem.*, 1895, cclxxxviii, 154.

The loss was due to the long purification. It would have been advisable to start with the pure heptose as this is easily purified.

The air dried substance did not lose in weight on heating to 76° in vacuum over P_2O_5.

0.1137 gm. of substance gave 0.1641 gm. CO_2 and 0.0796 gm. H_2O.

	Calculated for $C_7H_{14}O_7$ (210.13):	Found:
C	39.60	39.36
H	7.64	7.83

It crystallizes out of water and 70 per cent alcohol in needles and rosettes of needles. It is difficultly soluble in absolute alcohol. It softens at 138° and melts at 141–144°. The melt does not become entirely clear until the temperature 190° is reached. Owing to the small quantity of material available its rotation was not obtained.

d-α-Mannoheptaric Acid.

The calcium salt was prepared and purified according to Fischer's directions.[17] The twice recrystallized salt was decomposed with an equivalent amount of oxalic acid, neutralized with KOH, and evaporated to a syrup. On adding a little acetic acid and rubbing, the acid potassium salt crystallized out. Kiliani's[18] directions for *d*-α-galaheptaric acid were followed in converting this into the free acid. It melted at 168° (corrected). Kiliani gives 169° for *d*-α-galaheptaric acid.

Rotation.—Immediately after solution α = about −0.7°.

18 hours later $$[\alpha]_D^{30} = \frac{-1.13 \times 4.473}{1.02 \times 0.3012} = -16.5°$$

48 hours after solution $$[\alpha]_D^{30} = \frac{-1.23 \times 4.473}{1.02 \times 0.3012} = -17.9°$$

The rotation did not change after this.

Fischer found a final rotation of $[\alpha]_D^{20} = +15.08°$ for *d*-α-galaheptaric acid[19] but does not say how long he allowed the solution to stand.

[17] Hartmann, *loc. cit.*, 194.

[18] Kiliani, H., *Ber. d. deutsch. chem. Gesellsch.*, 1889, xxii, 522.

[19] Fischer, *Ann. d. Chem.*, 1895, ccIxxxviii, 155.

I also found that the acid potassium and the cadmium salts of *d*-α-mannoheptaric acid were similar to the corresponding salts of *d*-α-galaheptaric acid as described by Kiliani.[18] There does not seem to be any doubt that the two acids are antipodes.

Double Lactone of d-α-α-Mannooctaric Acid.

5 grams of pure *d*-α-α-mannooctonic acid lactone[20] were heated with 7.5 cc. of HNO_3, specific gravity 1.2, at 50° in a flask with air condenser. Crystals appeared in the solution in about three hours. After twenty-four hours these were filtered off and washed with a little water. Yield: 1.35 grams. They were recrystallized out of 15–20 cc. water. Yield: 1.06 grams. They were dried to constant weight over H_2SO_4 in a vacuum desiccator.

0.1827 gm. of substance gives 0.2752 gm. CO_2 and 0.0726 gm. H_2O.

	Calculated for $C_8H_{16}O_8$ (234.08):	Found:
C	41.01	41.08
H	4.31	4.45

The substance crystallizes out of saturated aqueous solution in colorless prisms, out of more dilute solution in hexagonal plates. The solution is neutral. It browns at about 250° and decomposes at 289° without melting.

0.2574 gram of substance was dissolved in 4.30 cc. N NaOH. After four hours a trace of phenolphthalein was added and it was found that 2.05 cc. N HCl were required to neutralize the solution. Since the 0.0011 gram molecule of lactone used required 0.00225 gram molecule of NaOH for neutralization, the substance is shown to be the double lactone of a dibasic acid. It reduces Fehling's solution strongly, but this does not necessarily indicate an aldehyde or ketone group, for the lactones of the sugar acids are known to have similar reducing properties. One of the best instances is the double lactone of mannosaccharic acid.

The final concentration of the solution was about 3.6 per cent. It showed no rotation in a 1 dm. tube under conditions where a rotation of 0.02° would have been perceived.

[20] Fischer and Passmore, *loc. cit.*, 2234.

RELATIVE TO THE TOTAL NITROGEN AND α-AMINO NITROGEN CONTENT OF PEPSINS OF DIFFERENT STRENGTHS.

PRELIMINARY COMMUNICATION.

By T. B. ALDRICH.

(*From the Research Laboratory of Parke, Davis and Company, Detroit.*)

(Received for publication, September 27, 1915.)

About a year ago, I secured a number of pepsins[1] of various strengths from our Digestive Ferment Department, for the purpose of determining what, if any, relation exists between the total nitrogen, the α-amino nitrogen, and the strength of the pepsins, for it was thought that this investigation might throw some light on the nature of pepsin in particular and of enzymes in general.

According to the pharmacopoeial tests the strength of the pepsins employed ran all the way from 1: 6,000 to 1: 15,000 in proteolytic activity; that is, they showed a wide variation in activity and should show some differences along the lines indicated.

The total nitrogen was determined by the official Gunning method.[2] Two blanks were made first with saccharose using the same reagents and in the same quantities used with the pepsin. Distillation and digestion were carried on for the same length of time in both cases.

The α-amino nitrogen was determined according to the method of Van Slyke,[3] using the larger apparatus. A 4 per cent solution of the pepsin was carefully prepared and 5 cc. of the solution (200 mg. of pepsin) were taken in most instances for each determination. Two determinations were always carried out, sometimes three or four.

[1] The pepsins were furnished me by Mr. Harvey Merker, head of the Digestive Ferment Department, and I wish to thank him heartily for the same.

[2] *U. S. Dept. of Agriculture, Bull. 108*, 1912, 7.

[3] Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 275.

Table I gives the results of the total nitrogen determinations; Table II those of the α-amino nitrogen; while Table III gives the average percentages of total nitrogen and α-amino nitrogen in the seven samples employed.

From the table and curve, it is seen that there is a gradual decrease in the percentage of α-amino nitrogen in the samples in the order of their strength. It would seem as though the method used in the purification of the pepsins gradually eliminates the simpler α-amino nitrogen compounds, and consequently causes an accumulation of more complex bodies in the stronger pepsins. Taking it for granted that with the still higher pepsins the recognizable α-amino nitrogen content will be further decreased, we would finally by sufficient purification obtain a pepsin having very little detectable α-amino nitrogen or an amount approximating that in the native protein, from which we could infer that the pepsins are of a more complex structure than the simpler

TABLE I.

Total Nitrogen in the Pepsins.

Pepsin used.	Amount of pepsin.	Acid used. $\frac{N}{10}$ H_2SO_4	Nitrogen obtained.	Nitrogen.
	gm.	*cc.*	*gm.*	*per cent*
No. 74 1:6,000	1.041	107.8	0.151	14.55
	1.095	113.2	0.159	14.57
A. P. B. 1:7,000	1.065	112.0	0.157	14.72
	1.035	108.5	0.152	14.68
	1.124	117.4	0.165	14.64
	1.530	121.0	0.170	14.70
No. 79 1:8,000	1.087	111.6	0.156	14.39
	1.064	108.7	0.152	14.30
No. 00 1:8,500	0.997	100.9	0.141	14.20
	1.026	102.4	0.143	13.98
	1.010	101.5	0.142	14.08
	1.082	109.2	0.153	14.10
No. 1 1:10,000	1.042	105.2	0.147	14.14
	0.995	100.5	0.141	14.15
No. 76 1:11,500	1.014	109.0	0.153	15.06
	0.975	103.4	0.145	14.85
No. 78 1:15,000	1.008	107.7	0.151	14.98
	1.006	106.9	0.151	14.90

TABLE II.

α-Amino Nitrogen in the Pepsins.

used.	Amount taken.	Nitrogen.	Temperature.	Barometer.	Nitrogen found.		Average.
	mg.	*cc.*	*°C.*	*mm.*	*per cent*	*mg.*	*per cent*
74 000	200	13.3	25	748	3.63	7.26	3.65
	200	12.4	25	748	3.39	6.77	
	200	14.2	26	748	3.86	7.71	
	160	11.0	26	748	3.73	5.97	
'. B. 000	200	13.5	30.5	744	3.55	7.11	3.55
	200	13.5	30.5	744	3.55	7.11	
79 000	200	12.5	25	744	3.39	6.79	3.40
	200	12.6	27	744	3.33	6.77	
	200	12.9	27	744	3.46	6.93	
	160	10.3	28	744	3.45	5.53	
00 500	200	11.8	28	744	3.14	6.28	3.08
	200	11.6	28	744	3.09	6.18	
	200	11.3	28	744	3.01	6.01	
'. 1),000	200	10.8	27	747	2.9	5.82	2.98
	200	12.1	28	747	3.2	6.48	
	160	8.6	29	746	2.86	4.58	
. 76 ,500	200	8.5	27	748	2.3	4.59	2.35
	200	8.6	27.5	748	2.4	4.63	
. 78 ,000	200	7.7	26	742	2.06	4.13	2.06
	200	7.9	26	742	2.12	4.24	
	200	7.5	25	742	2.00	4.01	

TABLE III.

No.	Strength of pepsin.	α-Amino nitrogen.	Total nitrogen.
		per cent	*per cent*
74	1:6,000	3.65	14.56
P. B.	1:7,000	3.55	14.69
79	1:8,000	3.40	14.35
00	1:8,500	3.08	14.09
1	1:10,000	2.98	14.15
76	1:11,500	2.35	14.96
78	1:15,000	2.06	14.94

Table I gives the results of the total nitrogen determinations; Table II those of the α-amino nitrogen; while Table III gives the average percentages of total nitrogen and α-amino nitrogen in the seven samples employed.

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	160	11.0	26	748	3.73	5.97	
A. P. B. 1:7,000	200	13.5	30.5	744	3.55	7.11	3.55
	200	13.5	30.5	744	3.55	7.11	
No. 79 1:8,000	200	12.5	25	744	3.39	6.79	3.40
	200	12.6	27	744	3.33	6.77	
	200	12.9	27	744	3.46	6.93	
	160	10.3	28	744	3.45	5.53	
No. 00 1:8,500	200	11.8	28	744	3.14	6.28	3.08
	200	11.6	28	744	3.09	6.18	
	200	11.3	28	744	3.01	6.01	
No. 1 1:10,000	200	10.8	27	747	2.9	5.82	2.98
	200	12.1	28	747	3.2	6.48	
	160	8.6	29	746	2.86	4.58	
No. 76 1:11,500	200	8.5	27	748	2.3	4.59	2.35
	200	8.6	27.5	748	2.4	4.63	
No. 78 1:15,000	200	7.7	26	742	2.06	4.13	2.06
	200	7.9	26	742	2.12	4.24	
	200	7.5	25	742	2.00	4.01	

TABLE III.

No.	Strength of pepsin.	α-Amino nitrogen.	Total nitrogen.
		per cent	*per cent*
74	1:6,000	3.65	14.56
A. P. B.	1:7,000	3.55	14.69
79	1:8,000	3.40	14.35
00	1:8,500	3.08	14.09
1	1:10,000	2.98	14.15
76	1:11,500	2.35	14.96
78	1:15,000	2.06	14.94

FIG. 1. The curve obtained, with the strength of the pepsins mar[illegible] along the abscissa at proportional intervals, and the percentages[illegible] α-amino nitrogen marked along the ordinate.

α-amino nitrogen compounds—that is, they approach the nat[illegible] proteins in complexity, where, according to Fischer's pept[illegible] theory of protein structure, they react with only a trace of th[illegible] nitrogen, nearly all of the latter being bound in the peptide li[illegible] ings of the protein molecule. In general the smaller the m[illegible] cules, the greater the proportion of free amino nitrogen, as [illegible] been indicated by the results with the peptides.

The higher pepsins having a strength of 1: 8,500 to 1: 15,0[illegible] contain from 3.08 to 2.06 per cent of α-amino nitrogen, equal [illegible] 13 to 20 per cent of the total nitrogen, which compares with t[illegible] α-amino nitrogen obtained by Van Slyke[4] for deutero-albumo[illegible] The pepsins may therefore have been either mixtures of nati[illegible] proteins and their hydrolytic products, or may have consist[illegible] entirely of such products of partial hydrolysis as the low[illegible] albumoses.

While the percentage of α-amino nitrogen shows a consta[illegible] though slight decrease with increased activity the percentage

[4] Van Slyke, *Jour. Biol. Chem.*, 1911, ix, 194.

total nitrogen in the samples shows very little variation, 14.14 to 14.94 per cent.

It it also interesting to note the following relative to the nitrogen content of *so called pure* pepsins obtained from different sources and by different methods and authors:

	per cent
Schoumow-Simanowsky[5]	14.55–15.00
Pekelharing[6]	14.13–14.75
Nencki and Sieber[7]	14.33
Bidder and Schmidt[8]	17.80
Chapoteaut[9]	15.4

[5] Schoumow-Simanowsky, E. O., *Arch. f. exper. Path. u. Pharmakol.*, 1894, xxxiii, 336.

[6] Pekelharing, C. A., *Ztschr. f. physiol. Chem.*, 1902, xxxv, 8.

[7] Nencki, M., and Sieber, N., *ibid.*, 1901, xxxii, 291.

[8] Bidder and Schmidt, Verdauungssäfte, Leipsic, 1852, quoted in Oppenheimer, C., Die Fermente, Leipsic, 1900, 97.

[9] Chapoteaut, P., *Compt. rend. Acad. d. sc.*, 1882, xciv, 1722.

CONCERNING THE IDENTITY OF THE PROTEINS EXTRACTED FROM WHEAT FLOUR BY THE USUAL SOLVENTS.

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Wheat flour was found by Osborne and Voorhees[1] to contain five distinct proteins: an albumin (leucosin), a globulin, a prolamin (gliadin), a glutelin (glutenin), and a proteose. The albumin is soluble in water, and both it and the globulin are soluble in dilute saline solutions. Gliadin is slightly soluble in water, and freely soluble in 50 to 70 per cent alcohol solutions. Glutenin is insoluble in water, saline solutions, and alcohol, but is dispersed by dilute acid and alkaline solutions. The existence in wheat flour of a proteose as such has been questioned. For the purposes of this work it has not been considered as present in appreciable quantities. The gliadin and glutenin constitute what is commonly known as gluten, and represent from 85 to 88 per cent of the total protein of a high grade flour. These are believed by Osborne[2] to be the only proteins present in the endosperm of the wheat kernel in any considerable amount. Ritthausen's conclusion that wheat flour contains three distinct proteins soluble in dilute alcohol was not supported by the work of Osborne, who found the fractional precipitations of the protein material soluble in alcohol to yield practically the same percentages of glutamic acid. In view of the similarity in the chemical and physical properties of these fractions, Osborne contends that only one alcohol-soluble protein is present. This view has since been generally

[1] Osborne, T. B., and Voorhees, C. G., *Am. Chem. Jour.*, 1893, xv, 392-471.

[2] Osborne, T. B., Proteins of the Wheat Kernel, *Carnegie Institution of Washington, Publication No. 84*, 108, 1907.

accepted. The chemical identity of gliadin from different wheat flours has since been demonstrated by the work of Wood,[3] Blish,[4] and Gróh and Friedl.[5] The albumin and globulin are contained chiefly in the embryo, and their presence in the flour is due to the impossibility of separating all of the germ from the endosperm during the process of roller milling.

These proteins are important not alone because of their nutritive value, but also because of their relation to the baking value of flour. It appears safe to postulate that there is no substance in wheat flour other than gluten which confers the property of retaining gases during fermentation to any marked degree. While the physical properties of the gluten complex may be affected by the presence of various electrolytes as suggested by Wood and Hardy,[6] and by the activity of proteoclastic enzymes, as shown by Baker and Hulton,[7] and Ford and Guthrie,[8] this complex must be present to give wheat flour dough its ability to expand and form a porous loaf. The marked differences in the physical properties of the two constituents of gluten, gliadin and glutenin, may be responsible for corresponding differences in the properties of flours in which they were present in varying proportions. The gliadin-glutenin ratio was held by Fleurent[9] and Snyder[10] to be of importance in estimating the baking strength of flours. Snyder later[11] stated that the percentage of gliadin in a flour is of more importance than the gliadin-glutenin ratio. Fenyvessy[12] found that the addition of gliadin to flour improved the baking quality, while added glutenin either had no effect or decreased the baking

[3] Wood, T. B., *Jour. Agr. Sc.*, 1907, ii, 139–161.

[4] Blish, M. J., *Jour. Ind. and Engin. Chem.*, 1915 (in press).

[5] Gróh, J., and Friedl, G., *Biochem. Ztschr.*, 1914, lxvi, 154.

[6] Wood, T. B., and Hardy, W. B., *Proc. Roy. Soc., Series B.*, 1909, lxxxi, 38–43.

[7] Baker, J. L., and Hulton, H. F. E., *Jour. Soc. Chem. Ind.*, 1908, xxvii, 368–376.

[8] Ford, J. S., and Guthrie, J. M., *ibid.*, 1908, xxvii, 389–393.

[9] Fleurent, E., *Compt. rend. Acad. d. sc.*, 1896, cxxiii, 755–758.

[10] Snyder, H., *Minnesota Agricultural Experiment Station, Bull.* 63, 1899, 519–533.

[11] Snyder, *Jour. Am. Chem. Soc.*, 1905, xxvii, 1068–1074.

[12] Fenyvessy, B. v., *Ztschr. f. Untersuch. d. Nahrungs- u. Genussmittel*, 1911, xxi, 658–662.

quality. Other investigations could be cited which support the same general conclusions.

The non-gluten proteins, albumin and globulin, were believed by Snyder[13] to play no important part in determining the bread-making qualities of flour. This was confirmed by Bremer.[14] The percentage of these proteins bears a relation to the grade of flour, since the lower grades contain larger proportions of bran and germ fragments, and therefore larger percentages of albumin and globulin. Considerable importance is accordingly attached to the development of accurate methods for the separation and estimation of the several proteins of wheat flour.

Gliadin has been quantitatively estimated in a number of ways. Fleurent[15] extracted the crude gluten with 70 per cent alcohol containing 2.5 to 3.0 grams of KOH per liter. The glutenin in the extract was precipitated by passing CO_2 through it until saturation was effected. An aliquot of the filtered extract was dried, and the weight of the dry matter less the potassium carbonate and bicarbonate present was considered to be gliadin. Fleurent later[16] suggested the use of a specially graduated densimeter for determining the percentage of gliadin extracted from crude gluten by 74 per cent alcohol.

Teller[17] proposed a scheme for the separation and estimation of the wheat proteins. He considered the proteose of Osborne to be gliadin, and accordingly recognized four proteins, gliadin, glutenin, leucosin, and edestin. Gliadin was determined by digesting the flour with hot alcohol, specific gravity 0.90 (66 per cent by volume). The nitrogen in the clear filtrate less the amide nitrogen was considered as gliadin nitrogen. Leucosin and edestin were extracted with 1 per cent NaCl solution, from which they were precipitated by adding sufficient alcohol to bring the concentration in the mixture to 75 per cent. The percentage of glutenin was calculated by difference. Teller determined the percentage

[13] Snyder, *Minnesota Agricultural Experiment Station, Bull. 54*, 1897, 37–42.

[14] Bremer, W., *Ztschr. f. Untersuch. d. Nahrungs- u. Genussmittel*, 1907, xiii, 69–74.

[15] Fleurent, *loc. cit.*

[16] Fleurent, *Compt. rend. Acad. d. sc.*, 1901, cxxxii, 1421–1423.

[17] Teller, G. L., *Arkansas Agricultural Experiment Station, Bull. 53*, 1898.

of nitrogen extracted by alcohol solutions ranging from 40 to 95 per cent. The largest quantity was found in the extracts when 40 to 55 per cent alcohol was used.

Snyder[18] suggested a polariscopic method for the estimation of gliadin in which 15.97 grams of flour were digested with 100 cc. of 70 per cent alcohol, and the filtrate was polarized in a 220 mm. tube. The reading on the sugar scale multiplied by 0.2 gave the approximate per cent of gliadin, the specific rotatory power of which is $-92°$. Matthewson[19] determined the optical rotation of gliadin in alcohols of various strengths, and in other organic solvents.

Chamberlain[20] found that 5 per cent K_2SO_4 solution extracted practically the same percentage of protein from flour as did 10 per cent NaCl, and proposed its use instead of the latter for the estimation of the albumin and globulin. On extracting flour with salt solution and the residue with alcohol, and *vice versa*, he found considerable quantities of the proteins which were soluble in one reagent to be extracted by the other. He later[21] reported the relative quantities of protein extracted by hot and by cold alcohol from the same flour, and found little difference in this regard, although the hot alcohol extracted slightly less protein. Snyder[22] reported the percentages of nitrogen in the extracts of flour with alcohol of 60 to 86.4 per cent by weight. The lower concentration of alcohol extracted the highest percentage of nitrogenous material in each case. He recommends the use of 70 per cent alcohol by weight, specific gravity 0.871. Ladd[23] as associate referee on Cereal Products for the Association of Official Agricultural Chemists proposed the use of alcohol of specific gravity 0.90 (66 per cent by volume) for the determination of gliadin, and extraction with 1 per cent NaCl solution,

[18] Snyder, *Jour. Am. Chem. Soc.*, 1904, xxvi, 263–266.

[19] Matthewson, W. E., *ibid.*, 1906, xxviii, 624–628 and 1482–1485.

[20] Chamberlain, J. S., *U. S. Dept. of Agriculture, Bureau of Chemistry, Bull. 81*, 1904, 118–125.

[21] Chamberlain, *Jour. Am. Chem. Soc.*, 1906, xxviii, 1657–1667.

[22] Snyder, *U. S. Dept. of Agriculture, Bureau of Chemistry, Bull. 105*, 1907, 88–90.

[23] Ladd, E. F., *U. S. Dept. of Agriculture, Bureau of Chemistry, Bull. 122*, 1909, 53–58.

followed by precipitation of the protein in the extract with phosphotungstic acid, for the determination of albumin and globulin. Robertson and Greaves[24] determined the refractive indices of gliadin in ethyl and propyl alcohols, acetone, phenol, acetic acid, and KOH solutions. Greaves[25] in a comprehensive investigation, studied the influence of a number of factors upon the quantitative determination of gliadin. He found that the per cent of nitrogen extracted with 70 and 74 per cent alcohol varied with the proportion of flour used. With the 70 per cent alcohol it was greater when 1.9963 grams of flour per 100 cc. were used than when the proportion was 15.97 grams per 100 cc. Increasing the period of extraction with 70 and 74 per cent alcohol from 24 hours to 48 hours did not materially affect the percentage of protein extracted. The maximum quantity of protein was in most instances extracted with 70 per cent alcohol, as compared with concentrations ranging from 60 to 80 per cent by volume. Hot 74 per cent alcohol extracted considerably larger percentages of protein than did cold 74 per cent alcohol, the nitrogen so extracted averaging 1.457 per cent and 1.290 per cent of flour, respectively. The specific rotation of the protein extracted with the hot alcohol was lower, however, being $-77.86°$, while that of the cold extraction was $-97.01°$. When the flour was first dried at 96°C. the percentages of nitrogen extracted by 74 per cent alcohol were diminished from an average of 1.290 per cent to 1.205 per cent. Greaves bases his recommendation that 74 per cent alcohol be employed in the determination of gliadin on his observation that there was less variation in the specific rotation of the protein extracted by it than when alcohols of other concentrations were used.

Hoagland[26] determined the percentage of protein extracted by alcohol of concentrations ranging from 10 to 75 per cent by weight. He found a gradual increase in the percentage of extracted nitrogen with alcohol solutions of between 10 and 50 per cent, followed by a decrease when more concentrated solutions were em-

[24] Robertson, T. B., and Greaves, J. E., *Jour. Biol. Chem.*, 1911, ix, 81–184.

[25] Greaves, J. E., *University of California Publications in Physiology*, 1910–15, iv, 31–74.

[26] Hoagland, R., *Jour. Ind. and Engin. Chem.*, 1911, iii, 838–842.

ployed. Raising the temperature of extraction with 50 per cent alcohol to 75° increased the percentage of extracted nitrogen from 1.18 per cent (in the cold) to 1.58 per cent. The use of 50 per cent alcohol by weight at room temperature is recommended for the determination of gliadin. Olson[27] states that 50 per cent alcohol extracts proteins other than gliadin, and that the latter can be separated as a coagulum by evaporating off the alcohol and heating in water. About 68 per cent of the total alcohol-soluble protein was thus coagulated. In a second paper Olson[28] states that 1 per cent NaCl solution extracts gliadin as well as leucosin and edestin, the gliadin constituting approximately 29 per cent of the total protein extracted. A method of separating the several proteins is outlined. Aliquots of the extract are boiled and evaporated to dryness, and the solid matter is digested with 55 per cent alcohol which is assumed to dissolve the gliadin and globulin, and leave the denatured albumin as an insoluble residue. The gliadin and globulin are separated by evaporating off the alcohol, and boiling in water, when the gliadin coagulates. This coagulum is separated from the uncoagulated protein by filtration, and the globulin in the filtrate precipitated by phosphotungstic acid.

These citations show the lack of agreement which exists as to the proper methods for the determination of the several proteins of wheat flour. The differences in the methods used are doubtless responsible in part for the conflicting statements regarding the proportions of each which are usually present, and their importance in estimating bread-making quality. It was with a view toward determining the character and strength of the solvents, and the conditions of extraction which would afford the most accurate separation and estimation of the proteins in this important food that the following experiments were undertaken.

EXPERIMENTAL.

A study of the chemical constitution of the several wheat proteins revealed the fact that there was a striking difference in the percentage of ammonia nitrogen in the products of hydrolysis by

[27] Olson, G. A., *Jour. Ind. and Engin. Chem.*, 1913, v, 917–922.
[28] Olson, *ibid.*, 1914, vi, 211–214.

HCl. This ammonia nitrogen, according to Osborne's postulate,[29] is in amide union in the protein molecule. Osborne and Harris[30] report the following ammonia fractions in the products of hydrolysis of the four wheat proteins:

Protein.	Total nitrogen.	Nitrogen as ammonia.	
		In protein.	Of total nitrogen.*
	per cent	*per cent*	*per cent*
Albumin	16.93	1.16	6.8
Globulin	18.39	1.42	7.7
Gliadin	17.66	4.30	24.3
Glutenin	17.49	3.30	18.9

* Our calculations.

Van Slyke[31] found the gliadin which he hydrolyzed to yield 25.52 per cent of its nitrogen as ammonia. The same percentage of ammonia nitrogen was found in the hydrolysate of gliadin prepared in this laboratory by Blish. It appeared that advantage might be taken of these marked differences to ascertain in how nearly a pure form the several wheat proteins were present in the extracts employed for their separation. If, as asserted by Olson, the alcoholic extract contained a mixture of about two-thirds gliadin and one-third globulin and albumin (either or both), the ammonia nitrogen in the hydrolysate would be decidedly reduced. Conversely the presence of 40 per cent of gliadin in the extract with 1 per cent NaCl solution would effect a marked increase in the ammonia nitrogen over what would be expected in a mixture of hydrolyzed albumin and globulin.

Accordingly wheat flour was extracted with the usual protein solvents in the manner described below, the preparation hydrolyzed for 20 hours with about 100 cc. of HCl, specific gravity 1.115, and the ammonia in the hydrolysate determined by vacuum distillation after the method of Van Slyke.[32] By observing cer-

[29] Osborne, The Vegetable Proteins, New York, 1912, 58.

[30] Osborne, T. B., and Harris, I. F., *Jour. Am. Chem. Soc.*, 1903, xxv, 353-353.

[31] Van Slyke, D. D., *Jour. Biol. Chem.*, 1911-12, x, 15-55.

[32] Van Slyke, *loc. cit.*, 20-21.

tain precautions, and maintaining a uniformity of procedure including concentration of acid used, length of time of hydrolysis, reduced pressure, a low temperature in the contents of the distillation flask, and time of distillation (30 minutes), uniform and accurate results were secured by this method. Sufficient material was used in all cases to afford enough protein to yield a considerable quantity of ammonia and total nitrogen, thus reducing the errors of determination and calculation.

The Salt-Soluble Proteins.

80 grams of a patent flour milled by the roller process from hard spring wheat (Laboratory No. B401) were extracted at refrigerator temperature with 4,000 cc. of 1 per cent NaCl solution. The mixture was shaken vigorously at intervals of 15 minutes for the first 3 hours, and then allowed to stand 17 hours. The supernatant liquid was passed through a folded filter, more than 3,800 cc. of clear filtrate being obtained. Aliquots were drawn for total nitrogen determinations. The remainder was evaporated until reduced to about one-fourth its original volume, and then dialyzed until practically free from salt. The material in the dialyzer was evaporated to dryness in a porcelain dish on the water bath, heated with HCl, washed into a flask, and hydrolyzed under a reflux for 20 hours. The hydrolysate was freed from HCl by vacuum distillation, the residue taken up with water, made to a measured volume, and aliquots were taken for the total, and the ammonia nitrogen determinations.

The flour residue after extraction with the 1 per cent NaCl solution was collected, pressed as free as possible from the solution, and dried in a vacuum oven at reduced pressure and a temperature of 40° to 43°C. The dried material was ground to the fineness of flour and reserved for further work.

The extract with this solvent contained 9.6 mg. of nitrogen per 100 cc., which is equivalent to 0.48 per cent of nitrogen on the basis of the original flour. The ammonia nitrogen in the hydrolyzed extract constituted 16.71 per cent of the total nitrogen. Since the globulin and albumin are present in the proportions of about 3 to 2, the weighted average of their ammonia nitrogen on hy-

drolysis would be approximately 7.3 per cent. The gliadin extracted by the 1 per cent NaCl solution is doubtless responsible for the increased ammonia fraction. A mixture of albumin and globulin in the proportions mentioned, with gliadin, in which the ammonia-nitrogen fraction on hydrolysis is 16.71 per cent, would consist of about 48.3 per cent of albumin and globulin, and 51.7 per cent of gliadin. It is evident, therefore, that the protein in the extract with this solvent is about half gliadin, and 1 per cent NaCl solution is of little value in the separation and estimation of the albumin and globulin in a flour.

80 grams of the same flour were then extracted with 4,000 cc. of 10 per cent NaCl solution. The same procedure was followed in extracting and hydrolyzing the extract, and the flour residue after extraction was saved and dried. 100 cc. of the clear extract contained 6.8 mg. of total nitrogen, equivalent to 0.34 per cent on the basis of the original flour. The ammonia nitrogen in the hydrolysate represented 10.37 per cent of the total nitrogen. Using the same method of estimation as before, the proteins in this extract consisted of 83.1 per cent of albumin and globulin and 16.9 per cent of gliadin.

The extraction was repeated, using 5 per cent K_2SO_4 solution as recommended by Chamberlain. 100 cc. of the clear extract contained 6.68 mg. of total nitrogen, equivalent to 0.334 per cent on the basis of the flour. The hydrolyzed extract yielded 9.89 per cent of the nitrogen as ammonia, which is equivalent to 85.7 per cent of albumin and globulin, and 14.3 per cent of gliadin. It accordingly appears that 10 per cent NaCl and 5 per cent K_2SO_4 solutions extract protein mixtures of practically the same character, in which the gliadin is present to the extent of only about one-sixth of the total protein, the other five-sixths being albumin and globulin. The data of these saline solution extractions are presented in tabular form in Table I. Column 4 of this table gives the estimated percentage of albumin and globulin nitrogen extracted from the flour, corrected by deducting the gliadin nitrogen present in the extract.

TABLE I.

Percentage of Total Nitrogen and of Albumin and Globulin Nitrogen Extracted with Saline Solutions.

Solvent.	Total nitrogen extracted.	NH_3 nitrogen in hydrolysate.	Estimated albumin and globulin nitrogen.	
			In proteins of extract.	In original flour.
1 per cent NaCl	0.480	16.71	48.3	0.232
10 " " NaCl	0.340	10.37	83.1	0.283
5 " " K_2SO_4	0.334	9.89	85.7	0.286

The Alcohol-Soluble Proteins.

The same patent flour was extracted with alcohol solutions of different concentrations to determine the purity of the gliadin extracted. The quantities employed were 20 grams of flour and 1,000 cc. of alcohol solution, and the time of extraction was 20 hours when conducted at room temperature. With 30 per cent alcohol by volume, 0.89 per cent of nitrogen was extracted. The ammonia fraction in the hydrolysate constituted 23.11 per cent of the total nitrogen. This indicated the presence of considerable albumin, or globulin, or both, in the extract, the estimated gliadin representing but 86.8 per cent of the total protein extracted. 50 per cent alcohol by volume extracted 1.27 per cent of nitrogen, and the ammonia nitrogen fraction on hydrolysis constituted 24.20 per cent of the total nitrogen. The gliadin thus extracted was evidently not mixed with as large proportions of other proteins as in the case of the 30 per cent alcoholic extract. It was estimated to constitute 92.9 per cent of the total proteins present. 70 per cent alcohol by volume extracted 1.14 per cent of nitrogen from the flour, and the ammonia nitrogen in the hydrolysate was 24.15 per cent. The relative proportion of other proteins mixed with the gliadin was about the same as in the 50 per cent alcoholic extract. The total quantity of gliadin extracted was less than when 50 per cent alcohol was used.

The experiment was repeated using 50 per cent alcohol, except that the extraction was conducted in pressure flasks which were heated in a water bath at 83° to 84°C. for 3 hours. This is the temperature at which the mixture boils in the air. The use of

pressure flasks precludes the possibility of loss of the solvent. The filtrate was slightly opalescent. The nitrogen in the extract represented 1.53 per cent of the original flour, a marked increase over that extracted by cold 50 per cent alcohol. On hydrolysis, 24.12 per cent of the nitrogen was present as ammonia, which is practically the same as that yielded by the cold 50 per cent alcoholic extract. This is equivalent to 92.4 per cent of gliadin. The actual gliadin nitrogen extracted was therefore 1.41 per cent, as compared with 1.18 per cent extracted by the same concentration of cold alcohol. This would indicate that hot alcohol should be employed to effect the complete extraction of this important constituent of wheat flour.

Table II gives the percentages of total nitrogen and gliadin nitrogen extracted by various concentrations of cold alcohol, and hot 50 per cent alcohol.

TABLE II.

Percentage of Total Nitrogen and of Gliadin Nitrogen Extracted with Alcohol Solutions.

Concentration of alcohol (by volume).	Temperature.	Total nitrogen extracted.	NH_3 nitrogen in hydrolysate.	Estimated gliadin nitrogen.	
				In proteins of extract.	In original flour.
	°C.				
30 per cent	22–25	0.89	23.11	86.8	0.771
50 " "	22–25	1.27	24.20	92.9	1.180
70 " "	22–25	1.14	24.15	92.6	1.056
50 " "	83–84	1.53	24.12	92.4	1.414

The reduction in the ammonia-nitrogen fraction in the hydrolysate from the alcoholic extractions was due to albumin, or globulin, for when the residue from the extraction with saline solutions, after drying and powdering, was extracted with 50 per cent alcohol and the extract hydrolyzed, the ammonia fraction was found to be 25.57 per cent. This is almost the identical ammonia-nitrogen fraction resulting from the hydrolysis of pure gliadin and indicates that the saline solution extracted the albumin and globulin completely. Since glutenin was present in this residue in considerable quantities and yet did not appear in the alcoholic extract in sufficient proportions to reduce the ammonia-nitrogen fraction

below that found for pure gliadin, it is evident that it is not appre ciably soluble in 50 per cent alcohol.

The residue from the extraction with 5 per cent K_2SO_4 solution was reextracted in pressure flasks with 50 per cent alcohol at 83° to 84° for 3 hours, to determine whether all of the gliadin was removed by this method. The residue from the alcoholic extraction was pressed free from the liquid, and hydrolyzed in the usual manner. The ammonia nitrogen in the hydrolysate constituted 18.6 per cent of the total nitrogen. This is so nearly the ammonia-nitrogen fraction reported by Osborne for pure glutenin *viz.*, 18.8 per cent, as to justify the conclusion that only glutenin remained after the hot alcoholic extraction, and that this method of extraction removed the gliadin completely. The deduction is also justified, based on the similarity in the degree of purity of the gliadin in the extracts with cold and hot 50 per cent alcohol, that the former does not extract all of the gliadin in the flour, nor does cold alcohol of the other concentrations used.

A low grade flour containing 0.615 per cent of nitrogen soluble in 5 per cent K_2SO_4 solution was extracted with 50 per cent alcohol to determine whether the higher percentage of albumin and globulin would result in larger quantities of these proteins being extracted by the alcohol. The alcoholic extract contained 1.2 per cent of nitrogen on the basis of the original flour. The hydrolyzed extract contained 24.28 per cent of its nitrogen in the form of ammonia, which shows the mixture of proteins to consist of about the same proportion of gliadin to non-gliadin protein as was in the alcoholic extract from the patent flour. This would indicate that the 50 per cent alcohol extracts a more or less constant quantity of non-gliadin protein, regardless of the percentage in the flour.

There is a marked difference in the relative proportion of gliadin in the 50 per cent alcoholic extract reported by Olson (69 per cent) and that found by us (93 per cent). This led us to investigate Olson's method for the separation of the gliadin from the non-gliadin protein. 40 grams of flour were extracted with 50 per cent alcohol, and the alcohol was evaporated from the clear filtrate the alcohol being replaced by water in which the coagulum was heated until a relatively small volume of liquid remained. The "alcohol-soluble non-coagulable" material was separated from

the coagulum by filtration, and hydrolyzed. The ammonia fraction in the hydrolysate represented 21.44 per cent of the total nitrogen. This shows the non-coagulable portion to be about three-fourths gliadin. It is evident therefore that a considerable quantity of gliadin escapes coagulation by the method recommended by Olson, which is responsible for the low gliadin values assigned the alcoholic extract by him.

SUMMARY.

The extract of a patent flour when 1 per cent NaCl is used as the solvent contained a large proportion of gliadin, representing more than half of the total protein extracted. The extract with 10 per cent NaCl and 5 per cent K_2SO_4 solutions contained only approximately 15 per cent of the protein as gliadin. The use of 5 per cent K_2SO_4 solution as suggested by Chamberlain is accordingly recommended for the determination of the non-gluten proteins.

Neither 30 per cent nor 70 per cent alcohol by volume extracted as high a percentage of protein as did 50 per cent alcohol, when the extractions were conducted at room temperature for 20 hours. The extraction of gliadin was not complete with any of these solvents unless the temperature was raised. Extraction with 50 per cent alcohol at 83° to 84° for 3 hours apparently effected a complete separation of the gliadin, and this method is recommended for this purpose. The proportion of gliadin to non-gliadin nitrogen in the extracts was the same whether the extraction was conducted at room temperature or 83°, in both instances representing approximately 93 per cent gliadin nitrogen. The quantity of non-gliadin proteins extracted by 50 per cent alcohol appears to be constant, regardless of the percentage of these constituents in the flour extracted.

The separation of gliadin from non-gliadin proteins by coagulation in water at the boiling temperature is not quantitative, considerable gliadin not being coagulated under those conditions.

A STUDY OF THE EFFECTS OF CERTAIN ELECTROLYTES AND LIPOID SOLVENTS UPON THE OSMOTIC PRESSURES AND VISCOSITIES OF LECITHIN SUSPENSIONS.[1]

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(Received for publication, September 4, 1915.)

Lecithin suspensions in water give characteristic colloidal solutions of the emulsoid type. They exhibit opalescence, and under normal conditions will remain in this state of colloidal solution indefinitely. It was thought that since lecithin and other lipoids are so widely distributed in living cells, a study of the influence of electrolytes and lipoid solvents upon these suspensions might lead to some results of physiological importance.

I. Osmotic Pressures.

A colloidal solution may be looked upon as a solution in which the colloidal particles are analogous to molecules, or, better, ions, of gigantic dimensions, each colloidal particle bearing an electrical charge, which in the case of lecithin is negative. This being the case colloidal solutions should, and do, exhibit osmotic pressures, the pressures depending upon the concentration and degree of dispersion of the colloid.

Since the particles are of comparatively large dimensions in any colloidal solution, it is an easy matter to obtain membranes which are permeable to crystalloids yet impermeable, or only slightly permeable, to colloids, thus enabling one to observe the effects of crystalloids upon the osmotic pressures of colloidal solutions. In an osmometer having such a membrane the crystalloid is free to pass through the membrane while the colloid is not.

[1] The author wishes to acknowledge his indebtedness to Prof. R. S. Lillie for suggesting this problem, and for his interest throughout the investigation.

Such an apparatus is described by R. S. Lillie,[2] and consists of a flask-shaped membrane of celloidin, into the neck of which is fitted a single-bored rubber stopper held firmly in place by an elastic band wound around it a sufficient number of times; the osmometer tube is fitted into the stopper.

The membranes are easily made from a solution of about 8 per cent celloidin in equal parts of alcohol and ether. Some of this solution is poured into a flask which has been previously dried by means of alcohol and ether, and rotated until an even layer covers the walls; then while the flask is still being rotated the excess is poured off. The solvent is removed by passing a current of air into the flask, and by washing with warm water. The temperature of the water should not exceed 25°C. at first but should be gradually increased until at the last washing it is about 50°C. The membranes can then be removed from the flask with ease after inserting a pipette between the flask and membrane, and blowing. If immersed in distilled water these membranes will remain in good condition for a week or two.

In filling the membranes a pipette having a stem sufficiently small to pass through the hole in the stopper was used, and the osmometers were filled, after having secured the stoppers in the membranes. The tubes having been inserted, the membranes were suspended in small battery jars covered with glass plates perforated by holes just large enough to permit the tubes to pass through.

The lecithin used in this investigation was that prepared from eggs and obtained from Merck. Suspensions were made by two methods: (1) by macerating the lecithin in warm water, and (2) by dissolving the lecithin in ether, then adding to water, and subsequently removing the ether by warming and passing a current of air through the solution. The latter method was much more convenient, yet care was necessary to prevent foaming caused by too vigorous shaking, or too great heating.

When first added to the water the ethereal solution of lecithin separates at the top, but gives a fairly fluid emulsion when shaken. Gradually as the ether is driven off the solution becomes homogeneous yet very viscous; soon, however, the viscosity begins to decrease and continues to decrease until all of the ether is removed, when a fairly fluid colloidal solution is obtained.

[2] Lillie, R. S., *Am. Jour. Physiol.*, 1907-08, xx, 133.

The osmotic pressures of 1 per cent lecithin suspensions, made by both methods, were measured. Little difference in osmotic pressure, if any, could be observed between the solutions prepared by the two methods. Different solutions, however, gave slightly different pressures; these differences are presumably due to the differences in the state of dispersion of the colloid.

The osmometer membranes used in the following experiments were of uniform size, all made in the same flask, and of about 30 cc. capacity; the osmometer tubes were of about 3 mm. bore; and the battery jars containing the outer fluid were of about 1,000 cc. capacity. All readings were corrected for capillary attraction.

The usual procedure was as follows. A 2 per cent stock solution for use in a series of experiments was made by the ether method. To 25 cc. of this solution sufficient of the lipoid solvent, or of a solution of the electrolyte, was added to give the desired concentration, and the resulting solution was diluted to 50 cc. In all cases the external medium had the same concentration of electrolyte, or of lipoid solvent, as the internal medium. When the concentration of the electrolyte is the same in both outer and inner fluids the pressure observed cannot be due to the electrolyte added but must be due to the colloid. Any change in pressure resulting from the addition of the crystalloid is due to the effect of the crystalloid upon the lecithin.

The pressures became constant after about forty-eight hours and the readings were then made. As the pressures were very low (a few mm. of water) a correction for temperature is unnecessary. All readings were made at room temperature (between 19°C. and 23°C.). The density may in all cases be regarded as unity. The results are given in millimeters, indicating the height of the solutions in the tubes after correction had been made for capillarity. As different stock solutions would naturally vary in degree of dispersion, controls were run with each series. Table I shows the osmotic pressures of 1 per cent suspensions of lecithin made by macerating in warm water.

Heating to boiling changes the osmotic pressures. Solutions 2a and 3a are Solutions 2 and 3 after having been removed from the membranes, heated to boiling, and then replaced in the os-

mometers. The external medium in the osmometer containing Solution 1 was changed once, but this caused no difference in the osmotic pressure.

TABLE I.

Osmotic Pressures of 1 Per Cent Lecithin Suspensions.

Solution.	Pressure.	Solution.	Pressure.
	mm.		mm.
1	2.0	4	2.1
2	2.0	2a	4.5
3	2.0	3a	4.3

Another 1 per cent solution was made in the same manner. With it were mixed the solutions already measured and tabulated in Table I. Portions of the resulting solution were put in different osmometers and the pressures recorded in Table II were observed.

TABLE II.

Osmotic Pressures of 1 Per Cent Lecithin Suspensions.

Solution.	Pressure.	Solution.	Pressure.
	mm.		mm.
1	6.8	4	7.2
2	7.0	5	7.0
3	7.0		

Immediately after introducing the solutions into the osmometers there was always a considerable initial rise. The pressures then gradually fell for about forty-eight hours, after which they remained practically constant. Table III shows the fluctuations in pressures of the solutions recorded in Table II.

After the osmometers have stood for some days a slight cloudiness usually appears in the external media, due to the escape of certain constituents of the inner solution. The quantity of material thus traversing the membrane is too small to affect the pressures appreciably. Upon long standing the solutions usually show a decrease in pressure, due probably to the change in the state of dispersion of the colloid. This change in the state of

dispersion will again be mentioned in connection with measurements of the viscosities.

TABLE III.

Fluctuations in Pressures of 1 Per Cent Lecithin Suspensions upon Standing.

Time after filling.	Solution 1.	Solution 2.	Solution 3.	Solution 4.	Solution 5.
hrs.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
½	36.2	36.8	31.8	39.0	45.4
24	7.8	7.8	8.4	8.8	10.5
48	6.8	7.0	7.0	7.2	7.0
48	External media changed				
3 days	External media again changed				
8 days	7.8	7.8	6.4	6.4	6.0

In Table IV are shown the osmotic pressures of four additional 1 per cent lecithin suspensions made (A) by macerating the lecithin in hot water, and (B) by the ether method. The solutions show no differences in pressures that can be attributed to the method of making.

TABLE IV.

1 Per Cent Lecithin Suspensions.

A. Made by macerating in hot water.		B. Made by ether method.	
Solution.	Pressure.	Solution.	Pressure.
	mm.		*mm.*
1	7.4	1	7.2
2	7.7	2	8.8

All of the electrolytes used were found to decrease the osmotic pressures of the lecithin suspensions. The pressures of the suspensions of 1 per cent lecithin made in various concentrations of different electrolytes are given in Tables V to IX. The external medium had always the same concentration with regard to electrolyte as the internal medium. The difference in pressure between the control and the solutions containing electrolytes, expressed in percentages, is also given.

TABLE V.

1 Per Cent Lecithin Suspensions in Solutions of Sodium Hydroxid[illegible].

Concentration of NaOH.	Pressure.	Difference.	Concentration of NaOH.	Pressure.	Differe[illegible]ce.
	mm.	*per cent*		*mm.*	*per c[illegible]n*
0	8.0		0	15.0	
N/800	5.5	−31.3	N/100	2.0	−86
N/400	2.0	−75.0	N/50	4.5	−70
N/200	1.8	−77.5	N/25	4.4	−70
N/100	1.9	−76.3	N/12.5*	—	—

* The solution at this concentration attacked the membrane.

TABLE VI.

1 Per Cent Lecithin Suspensions in Solutions of Sodium Chloride.

Concentration of NaCl.	Pressure.	Difference.	Concentration of NaCl.	Pressure.	Differen[illegible]ce.
	mm.	*per cent*		*mm.*	*per cen[illegible]*
0	8.4		0	9.5	
N/1,600	2.5	−70.2	N/100	1.0	−89.4
N/800	5.8	−30.9	N/50	1.2	−87.3
N/400	3.2	−61.9	N/25	2.0	−78.9
N/200	3.5	−58.3	N/12.5	1.3	−86.3
N/100	0.2	−97.6	N/6.25	1.0	−89.4

TABLE VII.

1 Per Cent Lecithin Suspensions in Solutions of Sodium Bromide.

Concentration of NaBr.	Pressure.	Difference.	Concentration of NaBr.	Pressure.	Difference.
	mm.	*per cent*		*mm.*	*per cent*
0	7.8		0	7.7	
N/3,200	3.0	−56.1	N/100	1.3	− 83.3
N/1,600	5.0	−35.9	N/50	0.9	− 88.4
N/800	1.2	−84.6	N/25	2.1	− 73.1
N/400	0.2	−97.4	N/12.5	0.9	− 88.4
N/200	5.7	−26.9	N/6.25	0.0	−100.0

TABLE VIII.

1 Per Cent Lecithin Suspensions in Solutions of Sodium Iodide.

ncentration of NaI.	Pressure.	Difference.	Concentration of NaI.	Pressure.	Difference.
	mm.	*per cent*		*mm.*	*per cent*
0	11.5		N/100	0.0	−100.0
r/1,600	6.5	−43.5	N/50	1.0	− 91.3
r/800	2.0	−82.6	N/25	—	—
r/400	1.0	−91.3	N/12.5	0.1	− 99.1
r/200	0.8	−93.1	N/6.25	1.5	− 86.9

TABLE IX.

1 Per Cent Lecithin Suspensions in Solutions of Hydrochloric Acid.

oncentration of HCl.	Pressure.	Difference.	Concentration of HCl.	Pressure.	Difference.
	mm.	*per cent*		*mm.*	*per cent*
0	15.7		N/100	1.0	− 92.8
r/1,600	4.3	−72.6	N/50	1.5	− 89.7
r/800	2.0	−86.5	N/25	0.0	−100.0
r/400	1.5	−89.7	N/12.5	0.0	−100.0
r/200	1.0	−92.8	N/6.25	0.0	−100.0

In Table X the osmotic pressures of lecithin suspensions in lutions of different lipoid solvents are given. The concentration lipoid solvent is the same for both internal and external media.

TABLE X.

Lipoid solvent and concentration. (Volume per cent.)	Pressure.	Difference.
	mm.	*per cent*
ntrol	14.8	
% ethyl alcohol	18.8	+31.4
% methyl alcohol	18.4	+28.6
% propyl alcohol (normal)	18.1	+26.6
% propyl alcohol (iso)	16.5	+15.4
% butyl alcohol	12.4	−13.3
% amyl alcohol	19.0	+32.8
.5% capryl alcohol	12.0	−16.1
.1% capryl alcohol	13.1	− 8.4
% ethyl ether	14.0	− 2.1

In the concentrations used all of the electrolytes were found to lower the osmotic pressures. On the other hand the lipoid solvents, with but three exceptions, increased the pressure. In the case of ether the absence of effect is probably to be explained by evaporation. Capryl alcohol is probably insufficiently soluble in water to produce marked effect, 0.5 per cent does not form a homogeneous solution. The pressures recorded in Table X are those observed after forty-eight hours, but after only thirty hours the pressures of all suspensions in solutions of lipoid solvents were observed to be greater than that of the control.

The membranes though soluble in alcohol showed no deterioration in these dilute solutions after the experiments had been completed.

In Table XI are compared the per cent differences in osmotic pressures due to the electrolytes in the different concentrations employed. These differences are all negative.

TABLE XI.

Concentration.	HCl	NaI	NaBr	NaCl	NaOH
N/1,600	72.6	43.5	35.9	70.2	—
N/800	86.5	82.6	84.6	30.9	31.3
N/400	89.7	91.3	97.4	61.9	75.0
N/200	92.8	93.1	26.9*	58.3	77.5
N/100	92.8	100.0	83.3	97.6-89.4	76.3-86.6
N/50	89.7	91.3	88.4	87.3	70.0
N/25	100.0	—	73.1	78.9	70.7
N/12.5	100.0	99.1	88.4	86.3	—
N/6.25	100.0	86.9	100.0	89.4	—
Average	91.6	85.8	75.2	74.4	72.9

* This discrepancy is unusual and is probably due to error.

It may easily be seen that the osmotic pressures are decreased[1] by the electrolytes in the general order HCl, NaI, NaBr, NaCl, NaOH, the greatest depression occurring with HCl.

[1] The decrease in osmotic pressure of colloids due to electrolytes has been observed by R. S. Lillie, Moore and Roaf, Pauli, and others. Lillie has discussed the matter at length in the *Am. Jour. Physiol.*, 1907-08, xx, 127.

II. Viscosities.

The viscosities of nearly all of the above suspensions of lecithin were measured after they had been removed from the osmometers. In some cases the viscosities were measured both before putting in and after removing from the membranes. The viscosities of the external media were measured after the pressures had been read; they are then slightly higher than pure solutions of the electrolytes of the same concentrations. This increase in viscosity is evidently due to the dialysis of certain components of the internal solutions.

All viscosities were measured at the same temperature, 25°C. An electrically heated thermostat was used which automatically remained constant within 0.03°C. The viscometer was allowed to remain in the thermostat sufficiently long to insure constant temperature throughout before measurements were made. A modified Bingham-White viscometer, such as is described by White,[4] was employed in this work.

In Tables XII to XVI the viscosities of the lecithin suspensions as well as the viscosities of the external media are given. In some cases the viscosities of the lecithin suspensions are given both before and after dialysis.[5]

TABLE XII.

1 Per Cent Lecithin Suspensions in Sodium Hydroxide Solutions.

Concentration of NaOH.	Viscosities.		Concentration of NaOH.	Viscosities.	
	Lecithin.	External media.		Lecithin.	External media.
0	0.01391	0.008963	0	0.01438	0.008953
N/1,600	0.01400	0.009066	N/100	0.01287	0.008958
N/800	0.01395	0.009041	N/50	0.01416	0.008966
N/400	0.01328	0.009032	N/25	0.01921	0.009032
N/200	0.01277	0.008951			
N/100	0.01256	0.008979			

[4] White, G. F., and Twining, R. H., *Jour. Ind. and Engin. Chem.*, 1913, v, 568. White, G. F., *Biochem. Ztschr.*, 1911, xxxvii, 482.

[5] The viscosity of pure water at 25°C. is 0.00895 in c.g.s. units.

TABLE XIII.

1 Per Cent Lecithin Suspensions in Sodium Chloride Solutions.

Concentration of NaCl.	Viscosities.		Concentration of NaCl.	Viscosities.	
	Lecithin.	External media.		Lecithin.	External media.
0	0.01421	0.009049	0	0.01271	0.009014
N/100	0.01299	0.009074	N/1,600	0.01253	0.009048
N/50	0.01175	0.009187	N/800	0.01377	0.009004
N/25	0.01130	0.009021	N/200	0.01282	0.009002
N/12.5	0.01104	0.009070	(The above solutions were measured		
N/6.25	0.01075	0.009127	after having been made 20 days.)		

TABLE XIV.

1 Per Cent Lecithin Suspensions in Sodium Bromide Solutions.

Concentration of NaBr.	Viscosities.		Concentration of NaBr.	Viscosities.	
	Lecithin.	External media.		Lecithin.	External media.
0	0.01318	0.009125	0	0.01301	0.008977
N/3,200	0.01249	0.008991	N/100	0.01319	0.009021
N/1,600	0.01352	0.009022	N/50	0.01305	0.009028
N/800	0.01217	0.009032	N/25	0.01244	0.009015
N/400	0.01368	0.009023	N/12.5	0.01191	0.009043
N/200	0.01308	0.008992	N/6.25	0.01183	0.009071

TABLE XV.

1 Per Cent Lecithin Suspensions in Sodium Iodide Solutions. Viscosities

Concentration of NaI.	Before dialysis.	After dialysis.	
	Lecithin.	Lecithin.	External media.
0	0.01520	0.01445	0.009109
N/1,600	0.01495	0.01252	0.009872
N/800	0.01509	0.01238	0.009043
N/400	0.01502	0.01460	0.009048
N/200	0.01443	0.01446	0.009078
N/100	0.01449	0.01408	0.009002
N/50	0.01428	0.01409	0.009095
N/12.5	0.01442	0.01279	0.009051
N/6.25	0.01248	0.01268	0.009075

TABLE XVI.

1 Per Cent Lecithin Suspensions in Hydrochloric Acid Solutions. Viscosities.

Concentration of HCl.	Before dialysis.	After dialysis.	
	Lecithin.	Lecithin.	External media.
0	0.01426	0.01316	0.008937
N/1,600	0.01417	0.01392	0.008975
N/800	0.01404	0.01411	0.008941
N/400	0.01407	0.01421	0.008922
N/200	0.01481	0.01584	0.008967
N/100	———	0.01701	0.008942
N/50	0.01426	0.01430	0.008975
N/25	0.01464	0.01440	0.008950
N/12.5	———	0.01444	0.009012
N/6.25	———	0.01851	0.009025

In general the electrolytes were found to lower the viscosities as well as the osmotic pressures of the lecithin suspensions. It will be noticed that the measurements of the solutions before introduced into the osmometers generally gave a higher viscosity than they did after removal. This is probably due to a change in the state of aggregation of the particles rather than to dialysis, for no more than a trace of substance—probably some impurity present in the lecithin preparation—dialyzes out of the membrane.

The decrease in viscosity is undoubtedly due to the action of the electrolyte on the lecithin, for in the concentrations used the electrolytes increase the viscosity of water. Since the membranes are permeable to electrolytes the effect of the electrolytes upon the viscosities of the lecithin suspensions may be indicated by the equation: $\frac{\eta_1 - \eta_2}{\eta_2} = x$, if η_1 represents the viscosity of the internal medium, η_2 that of the external medium, and x represents the change in viscosity. Here x represents the effect of the presence of lecithin on the viscosity of the solution; *i.e.*, the proportionate increase of viscosity resulting from the addition of lecithin to the solution of the electrolyte or lipoid solvent. The effect of the added substance (electrolyte or lipoid solvent) on the viscosity of the suspensions might also be represented thus:

$\frac{\eta_1 - \eta_2 \text{ (in presence of added substance)}}{\eta_1 - \eta_2 \text{ (in absence of added substance)}}$. The latter expression gives the ratio of that part of the total viscosity due to the lecithin in the presence of the electrolyte, or the lipoid solvent, to the part of the total viscosity due to the lecithin in the absence of those substances.

In the case of the increase of viscosity observed in the more concentrated solutions of sodium hydroxide and hydrochloric acid, the peculiar behavior may possibly be due to the direct combination of the lecithin, which is amphoteric, with those substances. The hydrochloric acid at the concentration of N/100 precipitates the lecithin markedly, less so at higher and lower concentrations (except at N/6.25), before introducing into the osmometers. After the solution had been in the osmometers for some time the precipitate appeared to reenter solution. It may be noticed that the viscosity increases as the concentration of the acid approaches N/100, falls again, and then rises as the concentration approaches N/6.25, where another precipitation point occurs.

Sodium hydroxide decreases the viscosity of the lecithin suspensions in concentrations up to N/100, above which the viscosity increases until the system forms a gel at a concentration of N/2. The viscosity of certain sodium hydroxide-lecithin suspensions increases with time. A series of observations showing this is given in Table XVII. The solution was allowed to remain in the viscometer, which was kept in the thermostat at 25°C. during the whole period of the experiment so that a temperature change factor could not enter.

TABLE XVII.

1 Per Cent Lecithin Suspensions in N/12.5 NaOH *Solution.*

Time.	Viscosity.	Time.	Viscosity.	Time.	Viscosity.
hrs.		*hrs.*		*hrs.*	
0	0.01348	3.0	0.01342	8.5	0.01513
0.5	0.01343	3.5	0.01343	21.5	0.02831
1.0	0.01331	4.5	0.01354	24.5	0.02926
1.5	0.01339	5.5	0.01377	31.5	0.02668
2.5	0.01337	6.5	0.01491	49.5	Plastic.

The viscosity decreases slightly at first, probably because of the action of the electrolyte before chemical combination takes place. Later as the reaction goes on the viscosity steadily increases. Bingham and others[6] have shown in certain cases that upon chemical union the viscosity increases, that is, the fluidities of associated compounds are not additive.

After thirty-one and one-half hours it was with difficulty that the solution began to flow through the viscometer; at this stage the solution had the consistency of a soft gel. Then as the structure of this gel was destroyed, by running through the capillary once, an even flow was obtained and the viscosity became somewhat lower than before. This decrease in viscosity is apparently due to the destruction of the structure of the gel. After forty-nine and one-half hours the gel had again set so that the state of plastic flow was reached and the viscosity could not be measured without again destroying the structure of the gel.

When a 1 per cent lecithin suspension in distilled water is allowed to stand the viscosity tends to decrease rather than increase. This change can probably be attributed to the change in the state of aggregation, for the viscosity of a colloidal solution depends upon both the concentration and the degree of dispersion of the particles. Table XVIII shows the decrease in viscosity of a 1 per cent lecithin suspension in distilled water upon standing.

TABLE XVIII.

Time.	Viscosity.	Time.	Viscosity.
hrs.		*hrs.*	
0	0.01361	0	0.01368
3.5	0.01358	5 days	0.01318
18.5	0.01320		

Table XIX shows the viscosities of different concentrations of lecithin in distilled water.

All solutions in Table XIX were made by diluting the 2 per cent solution and are therefore comparable.

[6] Bingham, E. C., White, G. F., Thomas A., and Cadwell, J. L., *Ztschr. f. physikal. Chem.*, 1913, lxxxiii, 641.

TABLE XIX.

Concentration.	Viscosity.
per cent	
Lecithin.	
0 (distilled water)	0.008950
0.1	0.009788
0.5	0.01108
1.0	0.01416
2.0	0.02547

It has been previously mentioned that during the preparation of lecithin suspensions, ether was found to increase the viscosity. The viscosities of 1 per cent lecithin suspensions in different concentrations of ethyl ether are given in Table XX. The concentration is given in volume per cent of lipoid solvent.

TABLE XX.

Concentration of ether.	Viscosity.	Concentration of ether.	Viscosity.
per cent		*per cent*	
0	0.01493	0	0.01567
0.10	0.01473	0.5	0.01639
0.25	0.01475	1.0	0.01664
0.50	0.01539	5.0	0.01817
1.00	0.01586	10.0	0.01909

In Tables XXI to XXVII the viscosities of 1 per cent lecithin suspensions in various concentrations of different lipoid solvents are given.

TABLE XXI.

1 Per Cent Lecithin Suspensions in Solutions of Ethyl Alcohol.

Concentration.	Viscosity.	Concentration.	Viscosity.
per cent		*per cent*	
0	0.01491	2.5	0.01645
0.10	0.01553	5.0	0.01717
0.25	0.01541	10.0	0.02131
0.50	0.01477	25.0	0.03140
1.00	0.01594	50.0	0.03332

TABLE XXII.

1 Per Cent Lecithin Suspensions in Solutions of Methyl Alcohol.

Concentration.	Viscosity.	Concentration.	Viscosity.
per cent		*per cent*	
0	0.01548	2.5	0.01615
0.05	0.01540	5.0	0.01736
0.10	0.01557	10.0	0.01925
0.25	0.01556	25.0	0.02490
0.50	0.01557	50.0	0.02501
1.00	0.01550		

TABLE XXIII.

Per Cent Lecithin Suspensions in Solutions of Normal Propyl Alcohol.

Concentration.	Viscosity.	Concentration.	Viscosity.
per cent		*per cent*	
0	0.01582	1.0	0.01643
0.05	0.01598	10.0	0.01826
0.10	0.01581	25.0	0.02007
0.50	0.01607	50.0	0.03385

TABLE XXIV.

1 Per Cent Lecithin Suspensions in Solutions of Isopropyl Alcohol.

Concentration.	Viscosity.	Concentration.	Viscosity.
per cent		*per cent*	
0	0.01597	1.0	0.01544
0.05	0.01601	10.0	0.02117
0.10	0.01559	25.0	0.02738
0.50	0.01662	50.0	0.03115

TABLE XXV.

1 Per Cent Lecithin Suspensions in Solutions of Capryl Alcohol.

Concentration.	Viscosity.	Concentration.	Viscosity.
per cent		*per cent*	
0	0.01402	0.50	0.01749
0.05	0.01526	1.00	0.01531
0.10	0.01493	5.00	0.01507

TABLE XXVI.

1 Per Cent Lecithin Suspensions in Solutions of Amyl Alcohol.

Concentration.	Viscosity.	Concentration.	Viscosity.
per cent		*per cent*	
0	0.01473	1.0	0.01633
0.05	0.01501	10.0	0.01650
0.10	0.01510	25.0	0.02721
0.50	0.01581		

TABLE XXVII.

1 Per Cent Lecithin Suspensions in Solutions of Butyl Alcohol.

Concentration.	Viscosity.	Concentration.	Viscosity.
per cent		*per cent*	
0	0.01493	0.50	0.01559
0.05	0.01512	1.00	0.01615
0.10	0.01512	10.00	0.01520

In Table XXVIII are shown the viscosities of the 1 per cent lecithin suspensions in solutions of lipoid solvents. These are the solutions of which the osmotic pressures are recorded in Table X.

TABLE XXVIII.

Lipoid solvent concentration.	Before dialysis.	After dialysis.		$\frac{\eta_1 - \eta_2}{\eta_2}$
	Lecithin.	Lecithin.	External media.	
10% ethyl alcohol	0.02013	0.01933	0.01191	0.623
10% methyl alcohol	0.01895	0.01820	0.01097	0.659
10% propyl alcohol (normal)	0.01953	0.02067	0.01239	0.668
10% propyl alcohol (iso)	0.01844	0.01893	0.01243	0.523
1% butyl alcohol	0.01626	0.01740	0.009246	0.863
0.5% capryl alcohol	0.01757	0.02125	0.008969	1.370
0.1% capryl alcohol	0.01735	0.02205	0.008930	1.469
1% amyl alcohol	0.01751	0.01816	0.009454	0.921
5% ethyl ether	0.01951	0.01548	0.009017	0.716
Control	0.01525	0.01436	0.008946	0.603

All of the solutions show an increase in viscosity due to the action of the lipoid solvent, with the exception of the isopropyl alcohol. The viscosity of this solution is low, however, compared with the value before observed and recorded in Table XXI.

f the value there recorded (0.02117) be used, an increase of 0.703 s obtained.

Alcohol and water mixtures have a viscosity greater than ither water or alcohol, as shown by Bingham and others;[7] but hen the equation before mentioned, $\frac{\eta_1 - \eta_2}{\eta_2} = x$, is applied, an ncrease in viscosity due to the action of the lipoid solvents upon he lecithin is noted.

Alcohol-water mixtures do not change in viscosity upon stand- ng, but suspensions of lecithin in alcohol-water mixtures do hange. Table XXIX shows such changes, which took place in 0 per cent ethyl alcohol solution.

TABLE XXIX.

1 Per Cent Lecithin Suspensions in 50 Per Cent Ethyl Alcohol Solution.

Time.	Viscosity.
Just made.....	0.03332
2 hrs. later.....	0.03370
24 " "	0.04240
5 days "	0.03515

After five days the viscosity had decreased, but no gelation vas noted with the lipoid solvents as with the sodium hydroxide olutions; apparently, however, some change in the composition of the mixture had taken place.

All of the viscosities given in this paper are the average of at least two measurements. Though the greatest care to insure accuracy was taken, the viscosities varied in a few cases nearly as much as 1 per cent for the same solution. Bingham and White,[8] in studying the viscosity and fluidity of emulsions, noted that at the critical solution temperature phenol-water emulsions behaved in the same manner. Their belief is that "in an emulsion, the fluidity is dependent upon the size of the drops and the dimensions of the apparatus, the viscosity being greatest when the drops are large in comparison with the cross-section of the capillary."

[7] Bingham, White, Thomas, and Cadwell, *loc. cit.*

[8] Bingham, E. C., and White, G. F., *Jour. Am. Chem. Soc.*, 1911, xxxiii, 1257.

The variation in viscosity of consecutive measurements was found most frequently in the case of lecithin suspensions in electrolyte solutions of low concentrations, the viscosity observed at the first measurement being generally greater than that of subsequent measurements. Taking into consideration the views of Bingham and White it appears that the emulsion is mechanically changed by forcing it through the capillary.

Bingham and Durham[9] in their paper on "suspensions of solids in liquids" have noted that electrolytes change the viscosity of suspensions when not present in quantities great enough to produce appreciable differences by themselves. They point out that viscosity measurements afford a delicate method of detecting changes in suspensions.

Schibig[10] points out that undissociated crystalloids increase the viscosity of solutions of organic colloids in direct proportion to the concentration of the crystalloid, while electrolytes generally decrease the viscosity.

Handovsky and Wagner[11] in working with lecithin suspensions found the viscosity to decrease when electrolytes were added, and to increase when narcotics were added. Their measurements, however, cannot easily be compared with the foregoing, for the results are not given in absolute units.

That the lipoid solvents, which are anesthetics, cause well marked changes in the viscosity of lecithin suspensions, and that in the case of ether at least, the viscosity decreases again after the removal of the lipoid solvent, are facts to be considered in the theoretical explanation of anesthesia from a physicochemical point of view; for the lipoids are widely distributed in nervous and other irritable tissues.

In conclusion it may be said that with but few exceptions the electrolytes studied decrease both the osmotic pressure and the viscosity of lecithin suspensions, while with the lipoid solvents an increase of both was observed. This change may be regarded as due to either a mechanical or a chemical alteration of the suspensions, or to both.

[9] Bingham, E. C., and Durham, T. C., *Am. Chem. Jour.*, 1911, xlvi, 278.
[10] Schibig, J., *Internat. Ztschr. physikal.-chem., Biol.*, 1914, i, 260.
[11] Handovsky, H., and Wagner. R., *Biochem. Ztschr.*, 1911, xxxi, 32.

THE ABDERHALDEN REACTION.

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Despite the tremendous amount of work which has been inspired by Abderhalden's idea of protective ferments, there has as yet been no indication of an approach to universal agreement concerning their specificity or utility for diagnostic purposes. The value of the "Abderhalden reaction," even for the detection of pregnancy, to say nothing of the diagnosis of less readily defined pathological conditions, is as much a matter of contention at present as when attempts to utilize the reaction first became general. The number of papers which have appeared both for and against the reliability of the reaction is so great that we shall attempt no review of the literature, most of which is cited in the bibliography of the latest edition of Abderhalden's "Abwehrfermente."

The present work was undertaken in the hope of providing for the measurement of serum protease a quantitative method sufficiently simple, accurate, free from subjective influence, and specific for proteolysis to afford definite conclusions concerning at least the facts of the Abderhalden reaction.

In attempting to ascertain the latter we have not investigated any of the applications of the Abderhalden reaction to pathology, but have confined ourselves to a study of pregnant compared with normal sera; because with these, according to the work of Abderhalden, one should certainly expect the most clear cut and

[1] Preliminary reports of the work here presented have been published in *Proc. Soc. Exper. Biol. and Med.*, Van Slyke, D. D., and Vinograd, M., 1914, xi, 154; Van Slyke, D. D., Vinograd, M., and Losee, J. R., *ibid.*, 1915, xii, 166.

reliable results. There is, furthermore, no chance for error in final confirmation of the diagnosis.

The method in most general use has been Abderhalden's "dialysis method," which, as described in the "Abwehrfermente," is so well known that description here is unnecessary. The fact that the method is not quantitative, and that even after the procedure has been successfully carried through all the preliminary details the final decision as to whether the result is positive or negative is based on the matching of colors, leaves the results peculiarly open to subjective influence on the part of the manipulator. Aside from this, the points for the possible introduction of errors are so numerous that it appeared possible, as Abderhalden has contended, that every failure by others to get good results with the dialysis method has been due to errors in technique.

The other method which has been chiefly utilized by the Abderhalden school has been the optical one, in which serum is incubated with peptone substrate, and the change in rotation observed in a polariscope. As the nature and optical rotation of the products are unknown, however, it is difficult to give a definite interpretation to the results. A technical difficulty lies, furthermore, in the slightness of the changes frequently observed. Being only a few hundredths of a degree, they often lie barely outside the possible limits of error in reading the instrument.

In the attempt to obtain quantitative results, Michaelis[2] utilized the familiar principle of measuring proteolysis by determining the non-coagulable nitrogen. After the serum had been incubated with substrate the proteins were precipitated by Michaelis and Rona's well known colloidal ferric hydrate method, and the amount of nitrogen in the filtrate was determined. Michaelis himself found the results by this method of as little diagnostic value as those he obtained by the dialysis procedure, but the colloidal iron method was later adopted by Abderhalden with excellent results. Other modes of coagulation have since been used, with varying success.

Another attempt to make the reaction quantitative was made during the past year by Thar and Kotschneff,[3] utilizing the amino nitrogen method

[2] Michaelis, L., and Lagermarck, L. v., *Deutsch. med. Wchnschr.*, 191[illegible] 4, xl, 317.

[3] Thar, H., and Kotschneff, N., *Biochem. Ztschr.*, 1914, lxiii, 483; 191[illegible] 5, lxix, 389.

vised by one of the writers. The serum was incubated with placenta ptone, and the resulting increase in amino nitrogen in the mixture termined. There was no definite difference between the results from rmal sera and those from pregnant sera. In other experiments, however, which placenta and serum were incubated in dialyzing thimbles and the ino nitrogen was determined in the dialysate, the results were negative th normal sera, but positive with nephritic as well as with pregnant sera. e protease of pregnant sera did not appear specific for placenta tissue, ce carcinoma and lung tissues were also digested.

Recently, on the other hand, Abderhalden has published a series of periments[4] in which a number of sera, pregnant, normal, and from anials in which specific ferments were supposed to have been generated by jection of proteins, were tested for specific proteases by all of the above ethods, as well as the interferometric method of Hirsch, and the same sults were obtained without exception by all the different means utilized. each case where the conditions were such as to indicate according to e theory that a specific protease was to be expected it was found, and in l other cases the substrates were not attacked at all.

As a possible standard method for measurement of serum otease the amino nitrogen determination seemed to us parcularly promising for the following reasons: First, it is *quantitive*, and permits accurate results with the small amounts of aterial available. Second, it is *specific for proteolysis;* it perits one to follow the chemical change which is characteristic of otein hydrolysis; *viz.*, the transformation of non-amino nitron in the $-CONH-$ peptide linkings into primary amino trogen as these linkings are hydrolyzed with the formation of $COOH$ and $-NH_2$ groups.

At first we utilized the simplest possible conditions. The bstrate, dried at room temperature at 0.5 mm. pressure over lphuric acid and then pulverized under sterile conditions, was cubated with the serum. After incubation the mixture was luted with a volume of water equal to that of the serum, centrigated, and the amino nitrogen content in 1 or 2 cc. was dermined by the micro-amino method of Van Slyke. The results ere compared with those from control portions of serum simirly treated in the absence of substrate.

Further experience showed that it was advantageous to remove ne proteins before determining the amino nitrogen. The al-

[4] Abderhalden, E., *Fermentforschung*, 1914, i, 20.

bumin and globulin of the serum contain, even when undigested, 8 and 5 per cent respectively of their total nitrogen in the form of free amino groups,[5] representing the ω-amino groups of the lysine molecules in the proteins. This amino nitrogen of the intact serum proteins amounted to three or four times the maximum which we observed formed as the result of the digestion with the substrate. The percentage accuracy with which the increase could be determined was consequently somewhat diminished by the amount of amino nitrogen present besides that due to digestion. Also the ω-amino group of lysine requires 15 to 30 minutes, according to the temperature, to react completely, while the α-amino groups liberated during digestion react in 3 to 4 minutes. In 5 minutes a definite proportion, from 80 to 90 per cent of the ω-nitrogen, according to the temperature, reacts with nitrous acid under the conditions of the determination; so that, by running the control determinations at the same temperature and for the same definite reaction period of 3, 4, or 5 minutes, increases due to liberation of α-amino groups by proteolysis can be measured with a fair degree of accuracy.

It is much more satisfactory, however, to remove the undigested proteins before determining the amino nitrogen of the digestion products, and we have found that the Michaelis colloidal ferric hydrate method affords an excellent means for accomplishing the removal. As the results by this technique are more accurate and the percentage increases in amino nitrogen greater than those observed when the proteins are present, it appears worth while to publish only the results obtained by the better method. We may state, however, that those obtained without removal of the proteins were of exactly the same significance as those given below.

Experiments with the Colloidal Ferric Hydrate Method in Preparing Serum for Amino Nitrogen Determination.

C. G. L. Wolf has already shown that Michaelis' colloidal ferric hydrate method is suitable for removing the proteins from blood in order to obtain a filtrate for quantitative determination

[5] Hartley, P., *Biochem. Jour.*, 1914, viii, 541. Van Slyke, D. D., and Birchard, F. J., *Jour. Biol. Chem.*, 1913–14, xvi, 539.

of free amino nitrogen.[6] In experiments on Witte peptone and partially digested proteins to be published later we have found, furthermore, that colloidal ferric hydrate not only lets all the amino-acids go through into the filtrate, but that it also precipitates none of the intermediary products up to the albumoses, and none of these except some of complexity but little below that of the original proteins (proportion of amino nitrogen was but 6 to 7 per cent of the total in the precipitated albumoses). As the precipitation of the native proteins themselves is complete, colloidal ferric hydrate appears especially well adapted to our purpose.

The following experiments show that closely agreeing results are obtained, even when the conditions of precipitation are not kept at all constant. In each case 2 cc. of normal horse serum, containing 7.5 per cent of protein, were diluted with 20 cc. of water and heated to boiling. The designated amount of Merck's colloidal ferric hydrate (containing 5 per cent of Fe_2O_3) was then added drop by drop. After a few seconds' boiling the magnesium sulphate, a solution made by dissolving $MgSO_4.7H_2O$ in an equal weight of water, was added to coagulate the excess of iron. The solution was then filtered through a folded paper into a 100 cc. Jena glass evaporating dish and washed four or five times with hot water. The filtrate was in every case except No. 7 water-clear. It was evaporated to dryness on the steam bath, the solution being transferred toward the end of the evaporation to a dish of only 4 or 5 cm. diameter. The dry residue was redissolved in 0.5 cc. of water, and transferred to the burette of the micro-amino apparatus. The apparatus had already been charged with nitrous acid and freed from air (first stage of amino nitrogen determination). The dish and burette were then washed with three more portions of 0.3 cc. each of water, and the apparatus was shaken 4 minutes, the determination being completed in the usual manner. The correction for the reagents was 0.040 cc. of gas. The corrected readings of nitrogen gas are given in the following table.

[6] Wolf, C. G. L., *Jour. Physiol.*, 1914, xlix, 89.

TABLE I.

No.	Serum.	Colloidal iron solution.	$MgSO_4$ solution.	Nature of precipitate and filtration.	Nitrogen [illegible] at 24°, 764 [illegible] mm., obtained [illegible] in amino nitr[illegible]ogen determinat[illegible]ion.
	cc.	*cc.*	*cc.*		*cc.*
1	2	3	1	Filtered clear but slowly. Precipitate bulky, apparently containing large excess of $Fe(OH)_3$	0.311
2	2	3	1	"	0.311
3	2	2	0.5	Filtered clear and more rapidly than 1 and 2. Precipitate less bulky	0.304
4	2	1	0.5	Ideal precipitation. Precipitate settled at once. Filtrate came through water-clear, and nearly as rapidly as hot water through an empty filter paper	0.313
5	2	1	0.5	"	0.310
6	2	1	0.5	"	0.306
7	2	0.5	0.5	Too little iron used. Protein not all precipitated. Solution foamed and clogged filter paper. Filtrate showed biuret and Heller's test	——

Preparation and Testing of Placenta Substrate.

We have utilized placentas prepared in three different ways: (1) According to Abderhalden's directions in every possible detail, and preserved in water under toluene. (2) Prepared practically the same as (1), but dried at room temperature and 0.5 mm. pressure, pulverized, and preserved dry in sterile bottles. (3) According to the method recently recommended by Pregl[7] as an improvement on Abderhalden's. The Pregl method, like (2), yields dry, pulverized material.

Abderhalden lays especial emphasis on the necessity for the careful preparation of the placenta substrate in a condition free from soluble nitrogen capable of giving the ninhydrin test for

[7] Pregl, F., *Fermentforschung*, 1914, i, 7.

-acids, free from hemoglobin, but not deprived of the te epithelial tissue, which appears to be or to contain the c substrate attacked by the serum protease. All of the ıtas were prepared with the strictest regard to the pre- d precautions. They were brought immediately from the ting room to the laboratory of the Lying-In Hospital, and ashing was begun while they were still warm. All used in ork reported here were prepared after considerable experi- had been gained during the preliminary work spent in ping the technique finally adopted for carrying out the reaction. Also, one of us, L., had had previous experience paring placentas for the Abderhalden reaction. We be- that our substrates met the requirements cited by Abder- ı as closely as extreme care and a fair amount of experi- ould render possible.

placenta tissues were tested at intervals for amino nitro- y digesting 0.1 gram of dry tissue, or as nearly as could be l an equivalent of the wet, with 2 cc. of water for 16 hours incubator, clearing the mixture with colloidal iron, and aining the amino nitrogen in the filtrate. In no case was a ıta used which yielded more than 0.01 cc. of nitrogen gas. ıe of the water extracts showed any trace of hemoglobin. ssue preparations themselves were quite white.

sterility, both of the substrates and of all the operations cted with the reaction, was occasionally controlled by plat- rum which had been digested with substrate in the usual r. Most of the plates were sterile after 48 hours' incu- . A few showed isolated colonies. We believe that the ıce of bacterial contamination on our results has been ed.

Pregl placentas (Class 3) as well as those prepared accord- Abderhalden met the tests described above. A large ıt of the tissue was lost as the result of the harsh mechanical ıent involved in the Pregl preparation, and the portion which ft both looked and behaved chiefly like resistant connective

The results obtained with it were different from those ed with the Abderhalden substrates only in that the Pregl tas gave smaller amounts of digestion products with both l and pregnant sera.

Details of Preparation of Placenta.—About 5 minutes after delivery, the placenta was placed in a basin with physiological salt solution and kneaded therein for the purpose of forcing the blood out of the vessels. Fresh portions of salt solution, alternated with distilled water, were added, about 2 liters being used for each washing. This kneading and washing continued until the placenta was nearly decolorized. After several washings, the placenta appeared as a pink colored mass, and the most difficult part of the work was to remove this color. This required many washings. Toward the end of the washing process, the blood vessels were carefully cut away from the placental tissue with small scissors. After the tissue was freed from blood vessels the washing was continued for some time, until the pink color disappeared entirely. The parts of the tissue which were not sufficiently decolorized were separated and thrown away. The whole washing period took from 3 to 4 hours.

Not all the placentas could be washed satisfactorily, as some of them retained some pink color even after numerous washings. Only the entirely decolorized placentas were used.

The coagulation of the proteins of the placenta and the extraction of amino-acids were accomplished by boiling the tissue in water slightly acidified with acetic acid. The amount of water used each time was about ten times the amount of placental tissue, and the boiling was repeated five or six times. The first time the boiling was continued for 30 minutes; the subsequent boilings were kept up for 5 minutes each.

The placenta prepared by the above method, together with some of the water used in the last boiling, was placed in a glass-stoppered bottle of such size that it was filled to about three-fourths of its capacity, and then sufficient toluene was added to completely fill it. The bottles were kept in an ice chest.

In Placentas 1 to 7 only, which were prepared in dry powder form, the Abderhalden method was varied as follows. The time of the washing process was considerably shortened by the use of 1 per cent solution of sodium citrate for the first washing. This prevented the blood from coagulating. From this stage on the usual method of washing was followed. After the last boiling, the water was carefully decanted and the tissues were rapidly dried in a desiccator under 0.5 mm. pressure, then pulverized under sterile conditions. The powder was kept in a sterile glass-stoppered bottle at room temperature.

In the preparation of the Pregl placentas Pregl's directions were followed entirely.

Details of the Serum Test.

The blood was allowed to stand for 3 to 4 hours in the sterile tube into which it had been drawn, in order to allow time for the clot to contract. The serum was then decanted off, centrifugated, and the clear serum removed with a pipette. The

erum was then submitted to a second centrifugation, in order o make certain that all formed elements were removed. epeated microscopic examinations failed regularly to reveal lls of any type in the serum after the second centrifugation. was also free from hemoglobin.

Of the clear serum 2 cc. measured with a bulb pipette were aced in a sterile tube with the substrate and covered with a yer of toluene 2 or 3 cm. deep. When dry pulverized placenta as used as the substrate 0.100 gram was weighed out on a sheet aluminum foil which had immediately before been sterilized a flame. When wet substrate was used, preliminary tests were ade to ascertain the approximate bulk of the substance which ontained 0.1 gram of dry material, and this amount was afterards taken, as nearly as could be judged by the eye, for incubation ith the serum. Care was taken that no pieces of substrate dhered to the sides of the tube above the toluene. Controls ere prepared in the same way, except that no placenta was added. he tubes were stoppered with sterile cotton and placed in the ncubator at 37° for 16 hours.

At the end of this time the contents were washed into a Jena eaker of 100 cc. capacity, about 20 cc. of water being used in the ansfer. The mixture was heated until it began to boil, then erck's colloidal ferric hydrate (containing 5 per cent of Fe_2O_3) as added from a pipette drop by drop, 1 cc. for the controls, cc. for the mixtures containing substrate. The liquid was boiled r 15 seconds or longer after all the colloidal ferric hydrate had een added, then 0.5 cc. of a 1 : 1 solution of crystalline magnesium lphate was added, and the boiling continued for another fraction of a minute. The precipitate was then allowed to settle, nd the solution decanted through a small folded filter into a Jena lass evaporating dish of about 100 cc. capacity. The precipitate as then washed several times, partly by decantation, partly n the filter, with hot water, the volume of the portions being so egulated that the combined filtrate and washings nearly filled he evaporating dish. The precipitate was granular, and could e washed so rapidly that both coagulation and washing were asily completed in five minutes. The washing removes every easurable trace of uncoagulated amino nitrogen. We repeatedly ubmitted precipitates to a repetition of the washing, evaporated

the filtrates from the second washings separately, and attempted to determine amino nitrogen in them. The results were always negative.

The filtrates obtained as above were evaporated on the steam bath until they were dry, or only a few drops of water remained. Standing on the bath for an hour after they had become dry did not appear to affect the amino nitrogen content of the residues, but the dishes were regularly removed from the bath within at most a half hour after the water had evaporated, and usually within a few minutes.

The quantitative removal of the redissolved residue to the micro-amino apparatus was rendered more easy by transferring the solution, after it had been concentrated to a few cc., to a smaller evaporating dish, and completing the concentration in that.

The final residue was redissolved in 0.5 cc. of water, with the aid of a slender, rubber-tipped stirring rod. The micro-amino apparatus[8] was then charged with nitrous acid and freed from air by two minutes' shaking (first stage of the amino nitrogen determination). The solution from the evaporating dish was then poured into the burette of the amino apparatus and the dish was washed with three successive portions of 0.3 cc. of water each, each portion being so guided by the rod during the transfer to the burette that the inner wall of the latter was washed down around the entire circumference. Each portion of the washing solution was admitted from the burette into the deaminizing chamber, so that the three portions used washed thoroughly both the evaporating dish and the burette, and transferred the entire serum residue to the deaminizing vessel of the amino apparatus. Less than a minute sufficed for the entire transfer, and it was performed in approximately the same time for the residues from the control tube and the tube containing placenta, so that the periods during which each was acted on by the nitrous acid should be as nearly as possible equal. As soon as the transfer was complete the apparatus was shaken for either 4 or 5 minutes, according as the temperature was above or below

[8] The form of apparatus used was that described in the note following this article.

20°. The determination was completed in the usual manner, and the volume of nitrogen read off in the gas burette.

Reference to Table I and to the duplicate controls in the tests with human serum shows that the errors accumulated during the entire manipulation seldom caused variation in the final result exceeding 0.01 cc. of nitrogen gas. The amounts of nitrogen gas (corrected for the reagents) from the controls varied from 0.18 to 0.28 cc. The presence of placenta substrate during the incubation caused increases usually between 0.05 and 0.20 cc. and sometimes over 0.25 cc. The changes observed were, therefore, many times greater than the experimental error.

It was thought that submitting the serum residues to acid hydrolysis, thereby changing peptones resulting from digestion of serum or substrate into amino-acids, might, through increasing the volume of gas obtained to measure, make the method still more sensitive. It was found, as a matter of fact, that the increases averaged about three times as great as those above mentioned, indicating that the average complexity of the proteolytic products in the colloidal iron filtrate was very roughly approximated, that of tripeptides. The results are given in Table II chiefly because this point may be of interest. For these determinations the residues were taken up in 20 per cent hydrochloric acid instead of water, and were heated in loosely stoppered tubes at 100° for 24 hours to hydrolyze the peptone. The solutions were then evaporated to dryness on the water bath, and used for amino nitrogen determinations. The addition of the hydrolytic treatment to the process, however, increased the error in the controls from 0.01 cc. to about 0.05 cc. of nitrogen gas. Consequently hydrolysis added nothing to the accuracy of the method, and the procedure adopted as the standard is the simpler one outlined in the previous paragraphs.

RESULTS.

The nature of the results is so readily apparent from inspection of the tables, and particularly of the charts, that discussion seems unnecessary. The conclusions which appear evident from them are stated in the following summary.

SUMMARY.

A simple and quantitative method has been established for measuring by amino nitrogen determination the extent of the proteolysis occurring when serum and substrate are incubated as in the Abderhalden reaction. The mixture after incubation is freed from protein with colloidal ferric hydrate, the filtrate evaporated, and the free amino nitrogen in it determined with the micro-amino apparatus. The increases in amino nitrogen observed when digestion occurs are many times greater than the experimental error of the method; so that it appears possible to rule out the latter as a factor in the results.

As controls, normal, not pathological, sera have been used; although as a point of independent interest, some determinations on pneumonic sera are reported.

Practically every serum, whether from a pregnant or a non-pregnant individual, showed protein digestion when incubated with placenta tissue prepared according to Abderhalden. The range of individual variation in proteolytic activity was wide. The range covered by most of the normal sera was, however, identical with that covered by the majority of the pregnant sera. As can be seen by reference to the charts, there is a tendency for the results from the pregnant sera to average somewhat higher than those from non-pregnant. The difference, even in the averages, is not great, however; and the individual variations of both pregnant and non-pregnant sera make the results from both overlap so completely as to render the reaction, even with quantitative technique, absolutely indecisive for either positive or negative diagnosis of pregnancy. The force of this statement is made apparent by even a cursory examination of the charted results.

Further evidence of non-specificity is seen in the fact that carcinoma tissue was digested to about the same extent as was placenta.

It appears that nearly all human sera can digest certain coagulated tissue proteins to some extent, but that the source and significance of the proteolytic agents, and the influences that cause their fluctuation, remain as yet undetermined.

We wish to express our appreciation to Dr. J. W. Markoe, Director of the Lying-In Hospital, for the assistance which he has afforded this work in placing the facilities of the Lying-In Hospital at our disposal; to Dr. C. F. Jellinghaus, to whom we are indebted for a portion of the material used in obtaining the results reported; to Dr. Isaac Levin for prepared carcinoma tissue; and to Dr. Cragin and Dr. Frederick Lyon of the Sloane Hospital for their courtesy in furnishing the material which rendered possible the preliminary work on the methods adopted.

EXPLANATION OF FIGURES.

The figures present graphically the extent of digestion observed with the different sera and substrates and expressed numerically in the last columns of the tables.

The abscissæ represent values of the difference (cc. of N_2 from 2 cc. serum incubated with placenta) − (cc. of N_2 from 2 cc. serum incubated alone); *i.e.*, the abscissæ give in terms of amino nitrogen the extent of protein digestion caused by the interaction of serum and placenta.

Results from normal male sera are indicated by ●
" " " female " " " " ⊕
" " pregnant sera " " " ×
" " pneumonic sera " " " □

The results obtained with each placenta are grouped between a pair of horizontal lines, each circle, cross, or square representing the result obtained with the serum of one individual acting on the placenta indicated. (In Table II results with one carcinoma tissue are also given.)

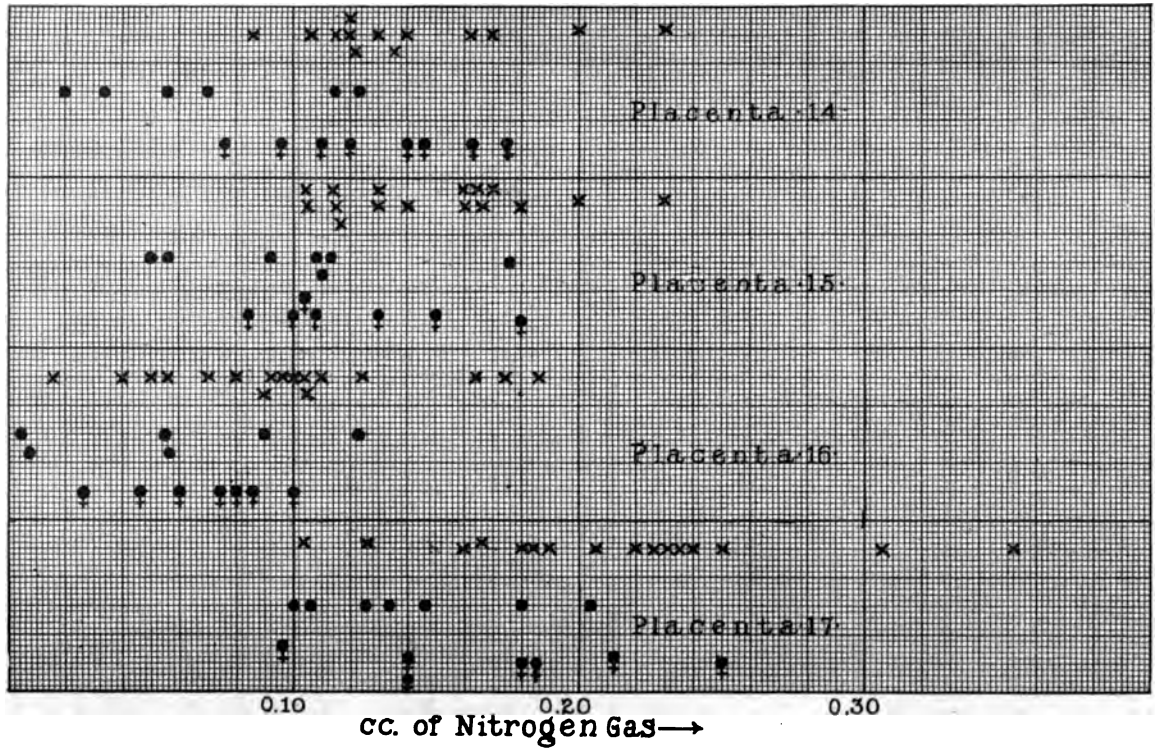

FIG. 1. (For explanation of symbols see p. 389.) Results from placentas prepared according to Abderhalden and preserved under toluene. With a given placenta it is seen that a majority of the results from both pregnant and non-pregnant sera fall within the same limits.

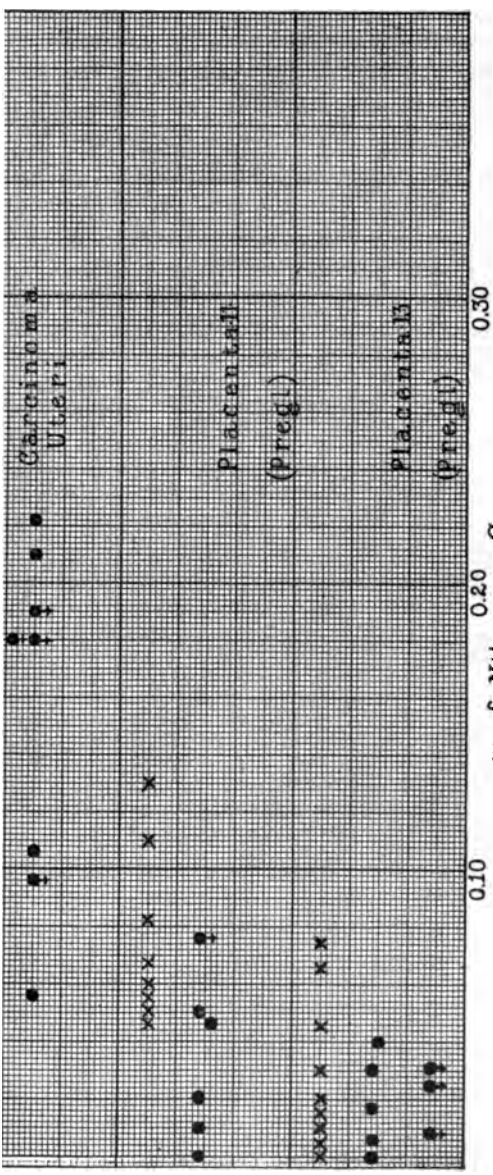

FIG. 2. (For explanation of symbols see p. 389.) Results from carcinoma tissue prepared according to Abderhalden and preserved dry, and from placentas prepared according to Pregl. Less digestion is observed with Pregl than with Abderhalden placentas, but the same non-specific results are obtained.

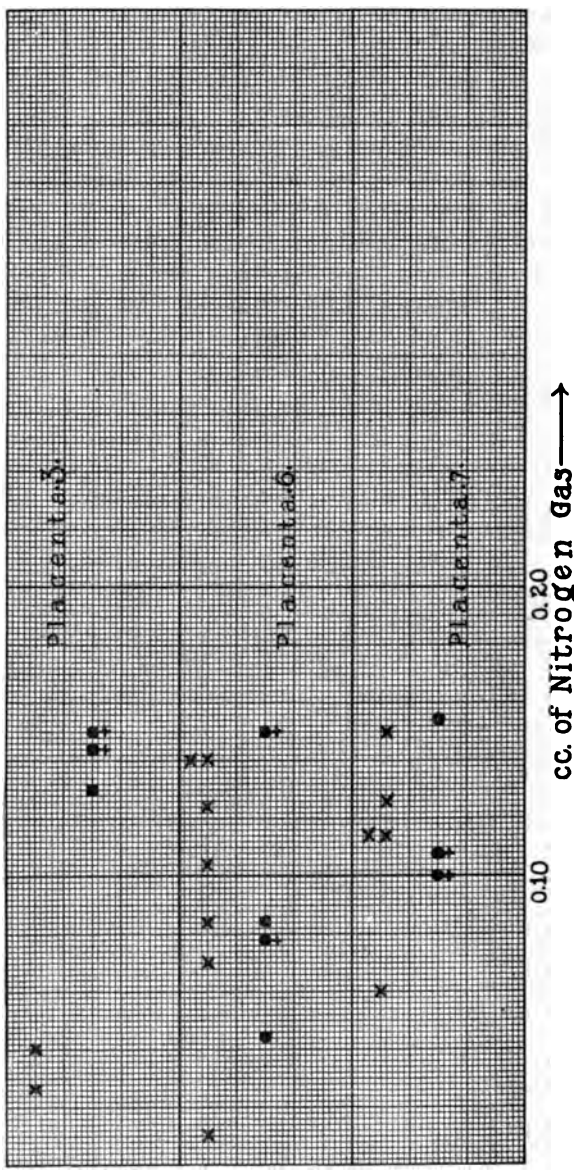

FIG. 3. (For explanation of symbols see p. 389.) Results from placentas prepared according to Abderhalden, except for initial washing with citrate, and preserved in dry powder form.

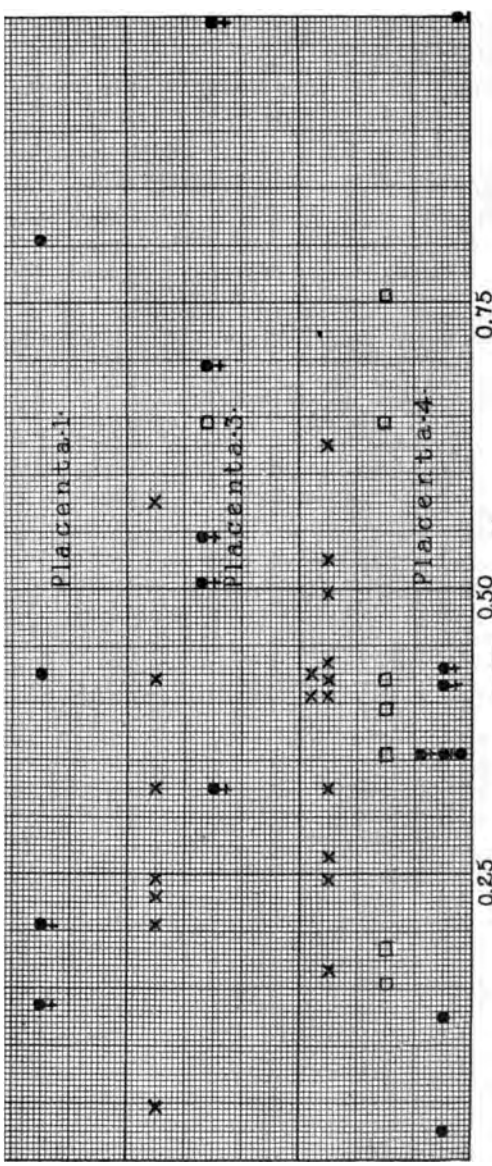

FIG. 4. (For explanation of symbols see p. 389.) Results from same class of placentas as in Fig. 3, but filtrates here were hydrolyzed before the amino nitrogen was determined. Comparison with Fig. 3 shows that hydrolysis about triples the increase in amino nitrogen in the serum filtrates, indicating that the average complexity of the "peptone" substances formed by the interaction of serum and placenta is about that of a tripeptide. Results are similar to those obtained without hydrolysis (other Figs.) in failing to show a marked or constant difference between pregnant and normal sera.

TABLE I A.

Normal Men. Standard Method (Filtrates Not Hydrolyzed).

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		cc.	cc.	cc.
1	Normal	2	—	0.25	—	—
		"	—	0.245	—	—
		"	—	0.255	—	—
		"	Placenta 6	0.33	0.08	0.08
2	Normal	2	—	0.275	—	—
		"	Placenta 6	0.39	0.115	0.115
		"	" 7	0.43	0.155	0.155
3	Normal	2	—	0.245	—	—
		"	—	0.255	—	—
		"	Placenta 6	0.32	0.07	0.07
		"	" 7	0.40	0.15	0.15
4	Normal	2	—	0.265	—	—
		"	Placenta 6	0.31	0.045	0.045
5	Normal	2	—	0.19	—	—
		"	—	0.185	—	—
		"	Placenta 6	0.27	0.083	0.083
		"	" 7	0.34	0.153	0.153
6	Normal	2	—	0.22	—	—
		"	Placenta 3	0.35	0.13	0.13
7	Normal	2	—	0.25	—	—
		"	—	0.245	—	—
		"	—	0.255	—	—
		"	Placenta 11	0.30	0.05	0.05
		"	" 12	0.31	0.06	0.06
		"	" 13	0.29	0.04	0.04
		"	" 14	0.32	0.07	0.07
		"	" 15	0.30	0.05	0.05
		"	" 17	0.35	0.10	0.10
8	Normal	2	—	0.275	—	—
		"	Placenta 11	0.28	0.005	0.005
		"	" 12	0.27	—	—
		"	" 13	0.27	—	—
		"	" 14	0.39	0.115	0.115
		"	" 15	0.45	0.175	0.175
		"	" 16	0.33	0.055	0.055
		"	" 17	0.40	0.125	0.125

TABLE I A—*Continued.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		*cc.*	*cc.*	*cc.*
9	Normal	2	—	0.28	—	—
		"	—	0.275	—	—
		"	Placenta 11	0.32	0.043	0.043
		"	" 12	0.30	0.023	0.023
		"	" 13	0.29	0.013	0.013
		"	" 14	0.33	0.053	0.053
		"	" 15	0.38	0.103	0.103
		"	" 16	0.33	0.053	0.053
		"	" 17	0.42	0.143	0.143
		"	Carcinoma uteri	0.38	0.103	0.103
10	Normal	2	—	0.245	—	—
		"	—	0.255	—	—
		"	Placenta 11	0.27	0.02	0.02
		"	" 12	0.275	0.025	0.025
		"	" 13	0.28	0.03	0.03
		"	" 14	0.33	0.08	0.08
		"	" 15	0.36	0.11	0.11
		"	" 16	0.34	0.09	0.09
		"	" 17	0.43	0.18	0.18
		"	Carcinoma uteri	0.46	0.21	0.21
11	Normal	2	—	0.265	—	—
		"	—	0.265	—	—
		"	Placenta 11	0.265	—	—
		"	" 12	0.260	—	—
		"	" 13	0.260	—	—
		"	" 14	0.30	0.035	0.035
		"	" 15	0.32	0.055	0.055
		"	" 16	0.27	0.005	0.005
		"	" 17	0.37	0.105	0.105
			Carcinoma uteri	0.32	0.055	0.055
12	Normal	2	—	0.255	—	—
		"	—	0.260	—	—
		"	Placenta 14	0.355	0.098	0.098
		"	" 15	0.35	0.093	0.093
		"	" 16	0.31	0.053	0.053
		"	" 17	0.39	0.133	0.133
13	Normal	2	—	0.19	—	—
		"	—	0.185	—	—
		"	Placenta 13	0.21	0.023	0.023
		"	" 14	0.31	0.123	0.123
		"	" 15	0.30	0.113	0.113
		"	" 16	0.31	0.123	0.123
		"	" 17	0.39	0.203	0.203
		"	Carcinoma uteri	0.408	0.221	0.221

TABLE I B.

Non-Pregnant Women. Standard Method.

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		cc.	cc.	cc.
14	Non-pregnant; breast abscess. Normal temperature Nov. 18. Blood was taken Nov. 24.	2	—	0.38	—	—
		"	Placenta 3	0.60	0.22	0.22
15	Uterus prolapse; positive Wassermann	2	—	0.44	—	—
		"	Placenta 3	0.59	0.15	0.15
16	Normal	2	—	0.11	—	—
		"	—	0.10	—	—
		"	Placenta 3	0.16	0.055	0.055
17	Normal	2	—	0.11	—	—
		"	Placenta 3	0.21	0.10	0.10
18	Normal	2	—	0.28	—	—
		"	Placenta 3	0.43	0.15	0.15
		"	" 6	0.43	0.15	0.15
		"	" 7	0.38	0.10	0.10
19	Normal	2	—	0.255	—	—
		"	Placenta 3	0.40	0.145	0.145
20	Non-pregnant; operated 7 days ago. Temperature normal	2	—	0.21	—	—
		"	—	0.195	—	—
		"	Placenta 6	0.28	0.078	0.078
		"	" 7	0.31	0.108	0.108
21	Miscarriage at 4 mos., 6 mos. ago. Blood taken 6 days after operation. Temperature normal	1.5	—	0.19	—	—
		"	Placenta 14	0.30	0.11	0.147
		"	" 15	0.27	0.08	0.107
		"	" 16	0.21	0.02	0.027
		"	" 17	0.35	0.16	0.213

TABLE I B—*Continued.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		*cc.*	*cc.*	*cc.*
22	Normal	2	—	0.20	—	—
		"	—	0.19	—	—
		"	Placenta 11	0.27	0.075	0.075
		"	" 13	0.22	0.025	0.025
		"	" 14	0.29	0.095	0.095
		"	" 15	0.28	0.085	0.085
		"	" 16	0.28	0.085	0.085
		"	" 17	0.38	0.185	0.185
23	Non-pregnant; delivered 5 mos. ago. Operated 7 days ago. Temperature normal	2	—	0.21	—	—
		"	—	0.195	—	—
		"	Placenta 13	0.21	0.008	0.008
		"	" 14	0.28	0.078	0.078
		"	" 15	0.31	0.108	0.108
		"	" 16	0.25	0.048	0.048
		"	" 17	0.30	0.098	0.098
		"	Carcinoma uteri	0.30	0.098	0.098
24	Normal	1.5	—	0.16	—	—
		"	—	0.155	—	—
		"	Placenta 14	0.28	0.123	0.163
		"	" 15	0.23	0.073	0.100
		"	" 17	0.26	0.103	0.140
		"	Carcinoma uteri	0.29	0.133	0.180
25	Normal	2	—	0.20	—	—
		"	—	0.20	—	—
		"	Placenta 13	0.23	0.03	0.03
		"	" 14	0.32	0.12	0.12
		"	" 15	0.33	0.13	0.13
		"	" 16	0.26	0.06	0.06
		"	" 17	0.34	0.14	0.14
			Carcinoma uteri	0.39	0.19	0.19
26	Never has been pregnant; normal	2	—	0.23	—	—
		"	—	0.23	—	—
		"	Placenta 14	0.37	0.14	0.14
		"	" 15	0.41	0.18	0.18
		"	" 16	0.33	0.10	0.10
		"	" 17	0.48	0.25	0.25

TABLE I B—*Concluded.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		cc.	cc.	cc.
27	Normal	2	—	0.28	—	—
		"	—	0.28	—	—
		"	Placenta 14	0.39	0.11	0.11
		"	" 15	0.43	0.15	0.15
		"	" 16	0.36	0.08	0.08
		"	" 17	0.46	0.18	0.18
			Carcinoma uteri	0.46	0.18	0.18
28	Normal	2	—	0.26	—	—
		"	—	0.25	—	—
		"	Placenta 14	0.33	0.075	0.075
		"	" 15	0.36	0.105	0.105
		"	" 16	0.33	0.075	0.075

TABLE I C.

Pregnant Women. Standard Method.

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		cc.	cc.	cc.
29	Pregnant 9 mos.	2	—	0.26	—	—
		"	—	0.26	—	—
		"	Placenta 3	0.30	0.04	0.04
30	Pregnant 9 mos.	2	—	0.24	—	—
		"	—	0.23	—	—
		"	Placenta 3	0.35	0.115	0.115
31	Pregnant 9 mos.	2	—	0.24	—	—
		"	Placenta 6	0.41	0.17	0.17
32	Incomplete abortion	2	—	0.34	—	—
		"	—	0.34	—	—
		"	Placenta 7	0.50	0.16	0.16
33	Pregnant 5½ mos.	2	—	0.20	—	—
		"	—	0.21	—	—
		"	Placenta 6	0.29	0.085	0.085
		"	" 7	0.32	0.115	0.115
34	Pregnant 5 mos.	2	—	0.23	—	—
		"	—	0.22	—	—
		"	Placenta 6	0.33	0.105	0.105
		"	" 7	0.34	0.115	0.115
35	Pregnant 6 mos.	2	—	0.17	—	—
		"	—	0.18	—	—
		"	Placenta 6	0.30	0.125	0.125
		"	" 7	0.30	0.125	0.125
36	Pregnant 6 mos.	2	—	0.18	—	—
		"	Placenta 6	0.32	0.14	0.14
37	Pregnant 9 mos.; eclamptic case	2	—	0.28	—	—
		"	—	0.28	—	—
		"	Placenta 6	0.42	0.14	0.14
28	Preeclamptic case	2	—	0.23	—	—
		"	Placenta 7	0.38	0.15	0.15

TABLE I C—*Continued.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		cc.	cc.	cc.
		2	—	0.20	—	—
39	Pregnant 9 mos.	"	Placenta 11	0.26	0.06	0.06
		"	" 13	0.265	0.065	0.065
		"	" 14	0.32	0.12	0.12
		"	" 15	0.36	0.16	0.16
		"	" 16	0.25	0.05	0.05
		"	" 17	0.44	0.24	0.24
		2	—	0.19	—	—
		"	—	0.18	—	—
40	Pregnant 9 mos.	"	Placenta 11	0.24	0.055	0.055
		"	" 13	0.21	0.025	0.025
		"	" 14	0.27	0.085	0.085
		"	" 15	0.30	0.115	0.115
		"	" 16	0.24	0.055	0.055
		"	" 17	0.32	0.135	0.135
		2	—	0.20	—	—
41	Pregnant 9 mos.	"	—	0.19	—	—
		"	Placenta 11	0.24	0.045	0.045
		"	" 13	0.20	0.005	0.005
		"	" 15	0.32	0.125	0.125
		"	" 16	0.21	0.015	0.015
		"	" 17	0.36	0.165	0.165
		2	—	0.23	—	—
42	Pregnant 9 mos.	"	—	0.235	—	—
		"	Placenta 11	0.290	0.068	0.068
		"	" 13	0.225	—	—
		"	" 14	0.355	0.123	0.123
		"	" 15	0.350	0.118	0.118
		"	" 16	0.330	0.098	0.098
		"	" 17	0.360	0.128	0.128
		2	—	0.29	—	—
43	Pregnant 9 mos.	"	—	0.285	—	—
		"	Placenta 11	0.37	0.082	0.082
		"	" 13	0.37	0.082	0.082
		"	" 14	0.45	0.162	0.162
		"	" 15	0.53	0.242	0.242
		"	" 16	0.41	0.122	0.122
		"	" 17	0.64	0.352	0.352

TABLE I C—*Continued*.

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		cc.	cc.	cc.
			—	0.20	—	—
44	Pregnant 9 mos.	2	—	0.20	—	—
		"	Placenta 11	0.33	0.13	0.13
		"	" 13	0.23	0.03	0.03
		"	" 14	0.37	0.17	0.17
		"	" 15	0.40	0.20	0.20
		"	" 16	0.305	0.105	0.105
		"	" 17	0.45	0.25	0.25
			—	0.18	—	—
45	Pregnant 9 mos.	2	Placenta 11	0.29	0.11	0.11
		"	" 13	0.20	0.02	0.02
		"	" 14	0.32	0.14	0.14
		"	" 15	0.29	0.11	0.11
		"	" 16	0.22	0.04	0.04
			—	0.18	—	—
46	Pregnant 9 mos.	2	—	0.185	—	—
		"	Placenta 13	0.18	—	—
		"	" 14	0.32	0.138	0.138
		"	" 15	0.35	0.168	0.168
		"	" 16	0.275	0.093	0.093
		"	" 17	0.42	0.238	0.238
		2	—	0.20	—	—
47	Pregnant 5½ mos.	"	—	0.21	—	—
		"	Placenta 13	0.22	0.015	0.015
		"	" 14	0.32	0.115	0.115
		"	" 15	0.31	0.105	0.105
		"	" 16	0.28	0.075	0.075
		"	" 17	0.395	0.190	0.190
		"	Carcinoma uteri	0.34	0.135	0.135
48	Pregnant 6 mos. (Examined)	2	—	0.20	—	—
		"	—	0.21	—	—
		"	Placenta 13	0.25	0.045	0.045
		"	" 14	0.31	0.105	0.105
		"	" 15	0.32	0.115	0.115
		"	" 16	0.31	0.105	0.105
		"	" 17	0.43	0.225	0.225
			Carcinoma uteri	0.36	0.155	0.155

TABLE I C—*Continued*.

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. serum.
		cc.		cc.	cc.	cc.
49	Pregnant 5 mos.	2	—	0.23	—	—
		"	—	0.22	—	—
		"	Placenta 13	0.23	0.005	0.005
		"	" 15	0.39	0.165	0.165
		"	" 16	0.29	0.065	0.065
		"	" 17	0.41	0.185	0.185
50	Pregnant 6 mos.	2	—	0.17	—	—
		"	—	0.18	—	—
		"	Placenta 13	0.25	0.075	0.075
		"	" 15	0.28	0.105	0.105
		"	" 16	0.36	0.185	0.185
		"	" 17	0.28	0.105	0.105
51	Pregnant 6 mos.	2	—	0.18	—	—
		"	Placenta 14	0.38	0.20	0.20
		"	" 15	0.36	0.18	0.18
		"	" 16	0.28	0.10	0.10
		"	" 17	0.43	0.25	0.25
			Carcinoma uteri	0.38	0.20	0.20
52	Pregnant 6½ mos.	2	—	0.20	—	—
			—	0.20	—	—
		"	Placenta 14	0.43	0.23	0.23
		1	" 15	0.18		0.16
		2	" 16	0.31	0.11	0.11
		"	" 17	0.36	0.16	0.16
		"	Carcinoma uteri	0.38	0.18	0.18
53	Eclamptic case, 9 mos. 6 convulsions	2	—	0.28	—	—
		"	—	0.28	—	—
		"	Placenta 14	0.41	0.13	0.13
		"	" 15	0.42	0.14	0.14
		"	" 16	0.37	0.09	0.09
		"	" 17	0.46	0.18	0.18
		"	Carcinoma uteri	0.43	0.15	0.15
54	Pregnant 9 mos.	2	—	0.21	—	—
		"	Placenta 11	0.26	0.05	0.05
		"	" 12	0.24	0.03	0.03
		"	" 13	0.22	0.01	0.01
		"	" 15	0.38	0.17	0.17
		"	" 16	0.29	0.08	0.08
		"	" 17	0.46	0.25	0.25

TABLE IC—*Concluded.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		cc.	cc.	cc.
55	Preeclamptic case; pregnant 9 mos.	2	—	0.22	—	—
		"	—	0.24	—	—
		"	Placenta 14	0.35	0.12	0.12
		"	" 15	0.36	0.13	0.13
		"	" 16	0.30	0.07	0.07
		"	" 17	0.45	0.22	0.22

TABLE II A.

Men and Non-Pregnant Women. Filtrates Hydrolyzed.

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		cc.	cc.	cc.
56	Woman, uterus prolapse; positive Wassermann	2	—	0.44	—	—
		"	—	0.46	—	—
		"	Placenta 3	1.00	0.55	0.55
		"	" 5	1.68	1.23	1.23
57	Woman, nonpregnant; normal	2	—	0.50	—	—
		"	Placenta 3	1.20	0.70	0.70
		"	" 5	0.91	0.41	0.41
58	Woman, nonpregnant; normal	2	—	0.35	—	—
		"	Placenta 1	0.55	0.20	0.20
		"	" 3	0.67	0.32	0.32
		"	" 5	0.70	0.35	0.35
59	Man, normal	1.4	—	0.29	—	—
		"	—	0.30	—	—
		"	Placenta 5	0.39	0.095	0.13
60	Man, normal	1.2	—	0.36	—	—
		"	Placenta 5	0.38	0.02	0.03
61	Man, normal	1	—	0.225	—	—
		"	Placenta 1	0.440	0.215	0.43
		"	" 5	0.350	0.125	0.25
62	Woman, normal	1	—	0.177	—	—
		"	Placenta 3	0.690	0.513	1.03
63	Woman, normal	1	—	0.29	—	—
		"	Placenta 1	0.36	0.07	0.14
		"	" 3	0.56	0.27	0.54
		"	" 5	0.50	0.21	0.42

TABLE II B.

Pregnant Women. Filtrates Hydrolyzed.

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 1 cc. of serum.
		cc.		cc.	cc.	cc.
64	Pregnant 9 mos.	2	—	0.36	—	—
		"	Placenta 5	0.80	0.44	.44
65	Pregnant 9 mos.	2	—	0.28	—	—
		"	Placenta 1	0.62	0.34	.34
		"	" 3	0.515	0.235	.235
66	Pregnant 9 mos.	2	—	0.26	—	—
		"	—	0.27	—	—
		"	Placenta 1	0.83	0.565	.565
		"	" 3	0.68	0.415	.415
67	Pregnant 9 mos.	2	—	0.26	—	—
		"	—	0.26	—	—
		"	Placenta 3	0.30	0.04	.04
		"	" 5	0.44	0.18	.18
68	Pregnant 9 mos.	2	—	0.26	—	—
		"	Placenta 5	0.50	0.240	.240
69	Pregnant 9 mos.	2	—	0.25	—	—
		"	Placenta 1	0.60	0.35	.35
70	Pregnant 9 mos.	2	—	0.20	—	—
		"	Placenta 3	0.44	0.24	.24
		"	" 5	0.46	0.26	.26
71	Pregnant 9 mos.	2	—	0.44	—	—
		"	—	0.42	—	—
		"	Placenta 5	0.59	0.16	.16
72	Pregnant 9 mos.	2	—	0.34	—	—
		"	Placenta 1	1.18	0.84	.84
73	Pregnant 9 mos.	2	—	0.41	—	—
		"	—	0.41	—	—
		"	Placenta 5	0.82	0.41	.41
74	Pregnant 9 mos.	2	—	0.24	—	—
		"	—	0.24	—	—
		"	Placenta 1	0.72	0.48	.48
		"	" 3	0.56	0.32	.32
		"	" 5	0.76	0.52	.52

TABLE II B—*Continued.*

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. serum.
		cc.		cc.	cc.	cc.
75	Pregnant 9 mos.	2	—	0.38	—	—
		"	—	0.42	—	—
		"	Placenta 1	0.68	0.28	0.28
76	Pregnant 9 mos.	2	—	0.34	—	—
		"	Placenta 1	0.60	0.26	0.26
		"	" 5	0.66	0.32	0.32
77	Pregnant 9 mos.	2	—	0.42	—	—
		"	—	0.38	—	—
		"	Placenta 3	1.02	0.62	0.62
		"	" 5	1.02	0.62	0.62
78	Pregnant 9 mos.	2	—	0.38	—	—
		"	—	0.36	—	—
		"	Placenta 5	0.86	0.49	0.49
79	Incomplete abortion	2	—	0.44	—	—
		"	Placenta 5	0.84	0.40	0.40
80	Pregnant 9 mos.; specimen was in ice box 4 days before experiment was started	2	—	0.20	—	—
		"	Placenta 5	0.62	0.42	0.42

TABLE II C.

Pathological Cases. Filtrates Hydrolyzed.

No.	Condition.	Amount of serum.	Substrate.	Nitrogen gas obtained.	Increase observed.	Increase per 2 cc. of serum.
		cc.		cc.	cc.	cc.
81	Man; pneumonia	2	—	0.53	—	—
		"	Placenta 5	0.68	0.15	0.15
82	Man; pneumonia	2	—	0.50	—	—
		"	Placenta 5	0.68	0.18	0.18
83	Woman; pneumonia	2	—	0.37	—	—
		"	Placenta 5	0.76	0.39	0.39
84	Man; pneumonia; very sick	2	—	0.56	—	—
		"	Placenta 5	0.91	0.35	0.35
85	Man; pneumonia; temperature normal, convalescent	2	—	0.41	—	—
		"	Placenta 5	1.05	0.64	0.64
86	Woman; pneumonia	2	—	0.44	—	—
		"	Placenta 3	1.03	0.59	0.59
87	Man; pneumonia	2	—	0.57	—	—
		"	Placenta 5	1.32	0.75	0.75
88	Man; pneumonia	2	—	0.38	—	—
		"	Placenta 5	0.80	0.42	0.42

NOTE ON THE MICRO-METHOD FOR GASOMETRIC DETERMINATION OF ALIPHATIC AMINO NITROGEN.[1]

By DONALD D. VAN SLYKE.

(From the Hospital of the Rockefeller Institute for Medical Research.)

(Received for publication, September 29, 1915.)

By slightly modifying the form of the gas burette the accuracy of the readings is so increased that the volume of the entire apparatus may be reduced to one-half that of the micro-amino apparatus described in our former paper (*i.e.*, so that only 1 cc. of solution is required for analysis), with corresponding reduction of the amount of material required to obtain results of the same percentage accuracy. The form of the burette is evident from the accompanying figure. The chief difference is that the zero point, instead of being placed at the bottom of the stopcock, is located on a capillary which extends for a few mm. below the cock. This permits marking off the upper boundary of the gas volume measured with an error of less than 0.001 cc. The burette, of 3 cc. capacity, is graduated into 0.01 cc. divisions about 1 mm. apart, so that by estimating tenths of a division gas volumes can be read to 0.001 cc. Such burettes must, of course, be accurately calibrated by the weight of water delivered.

A modification in the second stage of the determination (freeing the apparatus of air[2]) decidedly facilitates it. Instead of shaking back the nitrous acid mixture three times in the deaminiz-

[1] The principle of the method and the original form of the apparatus were described in *Jour. Biol. Chem.*, 1911, ix, 185. The apparatus in its present form, but requiring tenfold the amount of material was described in 1912, xii, 275. The application of the method to micro-analysis was described in 1913, xvi, 121. The present form of the apparatus, like those previously described, can be obtained from Emil Greiner, 55 Fulton St., New York.

[2] Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 279.

ing chamber, one needs to shake only once, until sufficient nitric oxide gas has been formed to force the liquid in the chamber down to the mark indicating the amount of nitrous acid solution that should be in the chamber when the amino solution is added. One then closes cock *a*,[3] and so turns cock *c* that gases from the chamber can escape from the outlet tube at *c*. The deaminizing vessel is then shaken two minutes rapidly with the motor. The nitric oxide evolved drives out the air as completely as it could

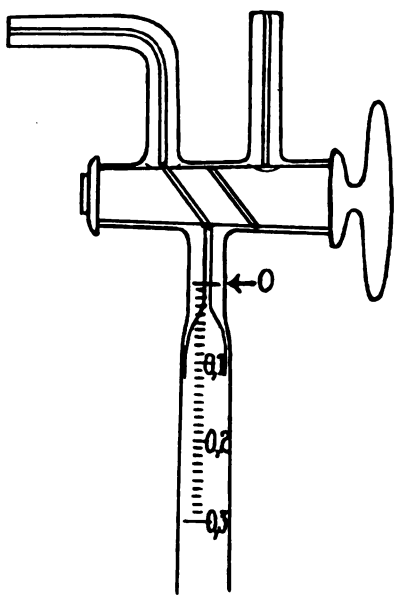

FIG. 1.

be removed by the originally described mode of operation; in fact, with the micro-amino apparatus the removal seems to be slightly more complete, and the operation is considerably simpler. After the two minutes' shaking the deaminizing vessel is connected through *c* with the gas burette, and the determination finished as previously described.

It may be mentioned that the rubber connections, particularly that joining the deaminizing vessel to the gas burette, should be

[3] See illustration, *Jour. Biol. Chem.*, 1912, xii, 278.

of soft, heavy-walled rubber tubing. We use "stethoscope" tubing, which is soft and flexible, and has a wall 3 or 4 mm. thick.

The results in the following table were obtained with a 1 per cent solution of Kahlbaum's synthetic leucine.

	N_2 gas.	Temperature.	Barometer.	Amino nitrogen.	
				Found.	Calculated.
	cc.	*°C.*	*mm.*	*mg.*	*mg.*
Solution measured in 1 cc. burette of apparatus. Burette correction = + 0.010 cc. Volume of solution analyzed = 1.01 cc. Weight of leucine = 10.10 mg.	1.957	25	757	1.081	1.080
	1.958	"	"	1.082	"
	1.957	"	"	1.081	"
	1.958	"	"	1.082	"
1.000 cc. of solution measured in calibrated Ostwald pipette and washed into deaminizing chamber	1.927	"	"	1.065	1.069
	1.932	"	"	1.068	"
	1.932	"	"	1.068	"

CORRECTION.

ANALYSIS OF PROTEINS BY DETERMINATION OF THE CHEMICAL GROUPS CHARACTERISTIC OF THE DIFFERENT AMINO-ACIDS.

By DONALD D. VAN SLYKE.

(From the Hospital of the Rockefeller Institute for Medical Research.)

On page 29 of the original article[1] the formula for calculating histidine should read

$$\text{Histidine N} = \frac{3}{2}\left(D - \frac{3}{4}\,Arg.\right)$$
$$= 1.5\,D - 1.125\,Arg.$$

In the original, the upper form of the equation

$$\text{Histidine N} = \frac{3}{2}\left(D - \frac{3}{4}\,Arg.\right)$$

is correct, but the lower is given as

$$\text{Histidine N} = 1.667\,D - 1.225\,Arg.$$

The error in the coefficient of D in the latter equation is obvious, but has previously escaped our attention because we have habitually used the correct upper formula.

[1] Van Slyke, D. D., *Jour. Biol. Chem.*, 1911-12, x, 29.

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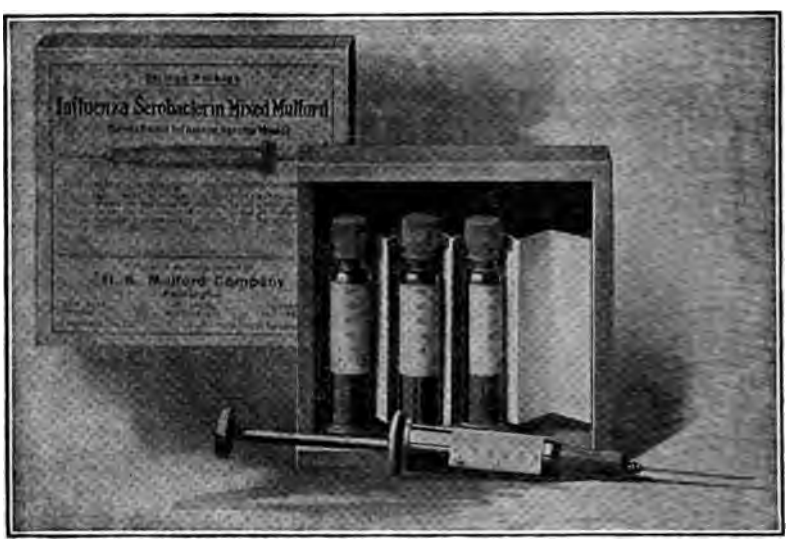

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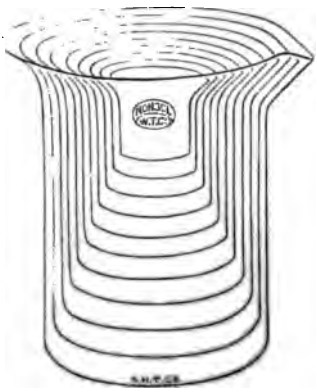

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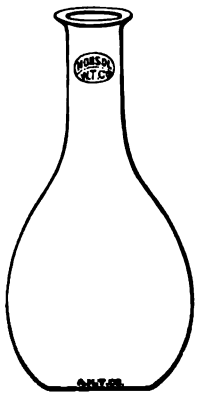

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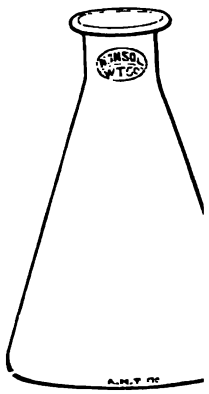

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STUDIES ON GROWTH.

II. ON THE PROBABLE NATURE OF THE SUBSTANCE PROMOTING GROWTH IN YOUNG ANIMALS.

By CASIMIR FUNK and ARCHIBALD BRUCE MACALLUM.[1]

(*From the Cancer Hospital Research Institute, London, and the Biochemical Laboratory, University of Toronto.*)

(Received for publication, October 8, 1915.)

Since the remarkable work of Osborne and Mendel,[2] Hopkins,[3] and McCollum and Davis,[4] who have shown that a young animal requires something special besides the usual food constituents for its process of growth, there has been much discussion as to the exact nature of this product. In our first paper[5] we discussed this subject and pointed out that most workers regard the growth factor as being closely associated with fats. The experimental evidence which led to this opinion was brought forward first by McCollum, but has become especially significant since Osborne and Mendel[6] and later Osborne and Wakeman[7] found that purified butter, which in their opinion was free from nitrogen, was still able to promote growth in young rats. In our earlier paper we found that even the purified butter contains traces of nitrogenous substances, and therefore might possibly contain traces

[1] The work was begun at the Cancer Hospital Research Institute, London, England; the experiments illustrated by the curves and tables were carried out in the Biochemical Laboratory, University of Toronto, during the tenure of a Senior Research Fellowship in the Department of Medical Research, and the expenses were defrayed by a grant from this department.

[2] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, Publication No. 156*, pts. i and ii, 1911.

[3] Hopkins, F. G., *Jour. Physiol.*, 1912, xliv, 425.

[4] McCollum, E. V., and Davis, M., *Jour. Biol. Chem.*, 1913, xv, 167.

[5] Funk, C., and Macallum, A. B., *Ztschr. f. physiol. Chem.*, 1914, xcii, 13.

[6] Osborne and Mendel, *Jour. Biol. Chem.*, 1913–14, xvi, 423.

[7] Osborne, T. B., and Wakeman, A. J., *Jour. Biol. Chem.*, 1915, xxi, 91.

of vitamines, an opinion which is shared by McCollum and Davis.[8] Recently MacArthur and Luckett[9] have found that the growth-promoting substance is not contained in the ether-soluble fraction, and they also suggest the possibility of vitamines as a factor.

To complete our first paper we carried out a series of experiments on young rats with ordinary butter and purified butter fat as the fat fraction of the diet. Both with butter and purified butter fat all the animals died after five to seven weeks, although on the former diet a slight initial advantage was noticed. Repeating these experiments on pigeons we convinced ourselves of the inability of both diets to prevent the onset of beri-beri symptoms, indicating the absence of or an insufficient quantity of beri-beri vitamine.

In our subsequent experiments we increased the percentage of butter and pure butter fat in the diets from 12 to 30 per cent with the same negative result in all cases. On both diets the rats showed, twenty-four to forty-eight hours before death, a condition of spastic contraction resembling somewhat avian beri-beri or infantile tetany.

We also carried out experiments in which increasing amounts of starch were replaced by equivalent quantities of unpolished rice, with lard as the fat fraction of the diet. The results, which were tending to support the vitamine theory of growth of one of us,[10] were still unsatisfactory, although the diet containing the largest percentage of unpolished rice proved to be much better both for maintenance and growth than those diets in which butter was used; yet this was finally inadequate as the animals declined after nine weeks.

Finally we made up diets, with butter used as fat, to which from 2 to 6 per cent of dried brewer's yeast was added. On this diet we have obtained successful growth and maintenance. As the growth-promoting factor is beyond question contained in yeast, we intend to fractionate the yeast and show which fraction contains the hypothetical growth substance.

The butter was purified as indicated in our earlier paper.[5] The casein was purified by extraction with hot alcohol. The fuel

[8] McCollum and Davis, *Jour. Biol. Chem.*, 1914, xix, 245.

[9] MacArthur, C. G., and Luckett, C. L., *Jour. Biol. Chem.*, 1915, xx, 161.

[10] Funk, C., *Ztschr. f. physiol. Chem.*, 1913, lxxxviii, 352.

value of the food and also the amount of the food absorbed from the intestine were controlled by means of an adiabatic calorimeter. The food mixture used, with the exception of the butter, was found to produce beri-beri in earlier experiments by one of us[11] on pigeons. Special experiments were carried out in order to ascertain the value of a food mixture consisting of casein, starch, cane-sugar, salt mixture, and butter for pigeons. All the pigeons fed on this food developed a typical beri-beri and no difference was noticed between the ordinary butter and the purified butter.

The results presented in this paper are a selection of a large number of experiments of uniform character. Two rats of the same sex were used in each experiment. The four diets used had the following composition.

Diet.	I.	II.	III.	IV.
	per cent	*per cent*	*per cent*	*per cent*
Casein	22	22	22	22
Sugar	10	10	10	10
Starch	33	33	31	27
Butter (ordinary)	30		30	30
(purified)		30		
Agar	2	2	2	2
Salt mixture*	3	3	3	3
Yeast (dry)			2	6

* The composition of the salt mixture was the same as in the experiments of Osborne and Mendel.[12]

Experiment I.—Chart I. The curves represent the average weight of two rats each of which was fed on Diet I (ordinary butter). As represented by the upper curve the rats showed a slight initial gain in weight and maintenance for about 20 days; then a rapid decline set in with fatal termination after 36 days. The lower curve represents the average weight of two rats which were changed from Diet I to Diet IV on the twentieth day. The effect of the addition of dried brewer's yeast was striking; the rats suddenly recovered and grew normally up to the end of the experiment. The intake of food and the absorption from the intestine are recorded below.

[11] Funk, *Ztschr. f. physiol. Chem.*, 1914, lxxxix, 373.

[12] Osborne and Mendel, *Jour. Biol. Chem.*, 1913, xv, 311.

D								
	gm.	*gm.*	*cal.*	*cal.*	*gm.*	*gm.*		
0	26.5				21.0			
4	32.25	25.1	120.5	3.67	27.5	18.15	89.2	3.09
8	36.5	19.6	95.4		26.5	15.0	74.7	
12	37.5	21.1	103.7	3.37	27.5	18.65	91.7	2.26
16	37.0	21.3	103.6		26.5	17.5	85.5	
20	33.0	23.0	125.1	2.66	27.0	25.65	134.2	5.42
24	30.5	21.5	111.2		38.0	43.2	229.5	
28	28.5	14.5	74.5	4.36	49.5	42.1	223.4	9.75
32	26.5	10.4	53.2		62.5	53.66	285.8	
36	23.5	10.2	52.2	2.23	73.5	57.95	308.8	16.69
40	Died				81.5	53.8	286.5	
44					85.0	54.15	288.5	
48					92.0	58.0	309.5	

This experiment was repeated on six male and two female rats with identical results.

Experiment II.—Chart II. Here we have used purified butter fat. Each curve represents the average weight of two male rats. The results are similar to those of the first experiment, only the maintenance period was slightly shortened. The same marked recovery was observed on changing from Diet II to Diet IV. The details of the experiment are recorded below.

	Upper curve, Rats 31 and 32.				Lower curve, Rats 29 and 30.			
Days.	Average weight.	Food intake.	Food.	Feces.	Average weight.	Food intake.	Food.	Feces.
	gm.	*gm.*	*cal.*	*cal.*	*gm.*	*gm.*	*cal.*	*cal.*
0	43.5				29.5			
4	49.0	32.2	176.8	8.34	36.5	25.85	141.2	4.25
8	52.0	26.35	143.9		39.5	18.3	99.9	
12	48.5	23.4	127.8	7.35	42.0	17.8	97.2	3.58
16	46.0	23.8	129.8		39.0	18.65	101.8	
20	44.5	23.75	129.6	8.09	36.0	17.75	96.9	3.43
24	42.0	24.1	131.5		31.5	17.65	96.6	
28	42.5	18.5	109.0	3.95	48.0	43.25	229.5	8.04
32	39.5	14.15	77.2		58.5	54.45	290.2	
36	36.5	11.85	68.2		74.5	61.85	324.3	13.09
40	32.5	6.70	36.79		86.0	53.5	285.0	
44	Died				94.5	57.1	304.2	
48					103.0	61.70	328.8	

[illegible] experiment was repeated on seven male rats and one female wi

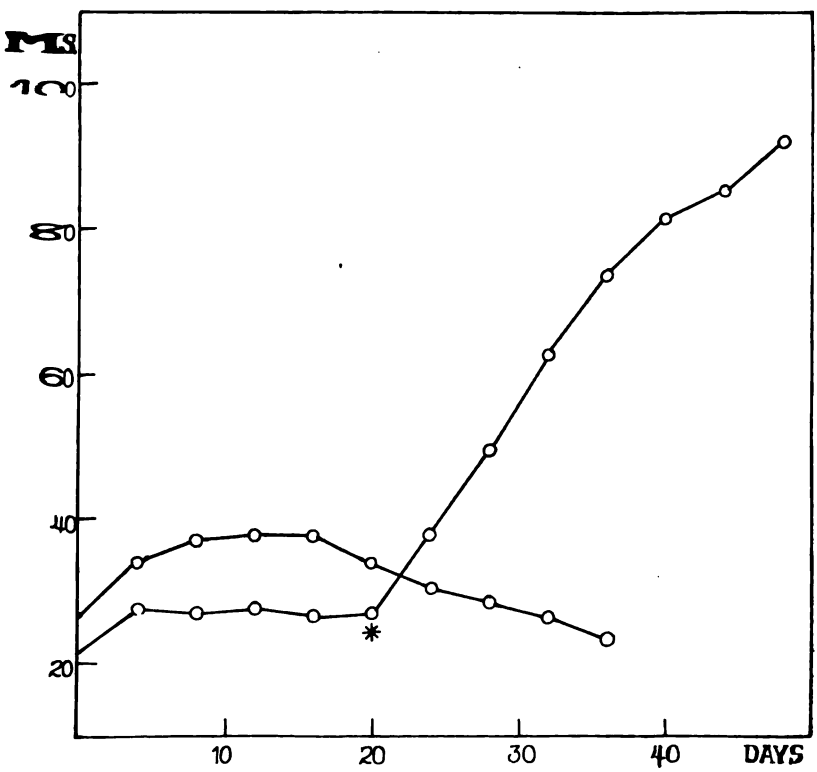

FIG. 1. Upper curve, Rats 25 and 26. Lower curve, Rats 27 and 28. On the curve at the point (*) Diet I was changed to Diet IV; Rats 25 and 26 died after 36 days on Diet I.

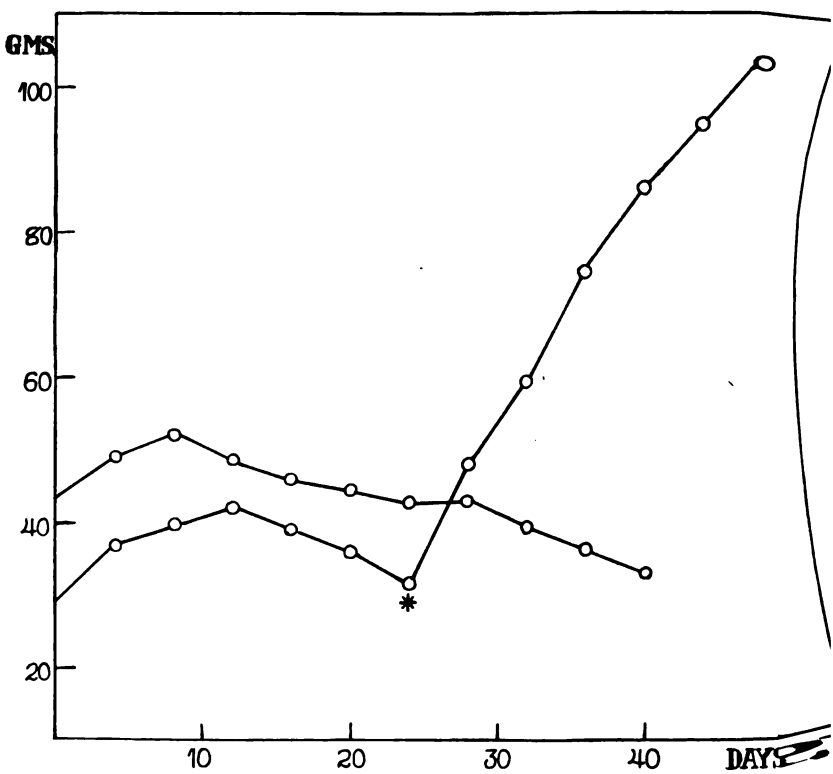

FIG. 2. Upper curve, Rats 31 and 32. Lower curve, Rats 29 and 30. At the point (*) on lower curve Diet II was changed to Diet IV. Rats 31 and 32 died after 40 days on Diet II.

Experiment III.—Chart III. In this experiment the upper curve illus-es the weight of two male rats, and the lower curve the weight of female rats. Up to 20 days Diet III was used (with 2 per cent st), being then replaced by Diet IV. In one set this was continued to end of the experiment (lower curve). In the other experiment Diet was replaced after 32 days, by Diet II (without yeast); the growth ed abruptly and the animals rapidly declined. No marked differ-e was noticed between the diets containing 2 and 6 per cent of yeast. details of the experiment are recorded below.

Upper curve, Rats 33 and 34.				Lower curve, Rats 35 and 36.			
Average weight.	Food intake.	Food.	Feces.	Average weight.	Food intake.	Food.	Feces.
gm.	*gm.*	*cal.*	*cal.*	*gm.*	*gm.*	*cal.*	*cal.*
20.0				18.5			
29.0	29.1	151.2	5.31	27.5	26.2	136.2	3.41
35.0	23.05	117.3		32.0	19.4	97.9	
37.0	25.95	132.4	7.30	37.0	26.65	137.1	4.39
41.5	26.60	137.2		39.5	29.85	148.1	
43.5	30.85	161.5	6.45	42.5	34.15	178.7	7.44
47.0	41.15	217.5		49.5	44.6	236.8	
59.0	44.0	233.5	8.06	58.0	43.5	230.9	11.13
69.0	49.5	262.6		69.5	48.75	259.5	
72.5	41.05	225.5	9.53	80.0	60.70	323.5	13.68
74.5	34.15	187.7		87.5	56.70	302.2	
71.0	30.85	169.6		90.0	51.90	276.5	
68.5	32.90	180.9		94.0	58.25	310.4	

This experiment was repeated with two additional female rats with same result.

Our new series of experiments clearly show the absolute ina-ty of either butter or purified butter fat to stimulate the growth young rats. This result could only be expected from our ex-iments with the same diet on pigeons, which have shown the ence of vitamine in butter. From the tables we see a strict tionship between the growth observed and the food taken in. also notice that the addition of yeast stimulates, directly or irectly, the appetite. The calorific determination of the food feces shows in all cases a practically complete absorption of food by the digestive tract, as the calorific value of the feces resents but from 2.5 to 5 per cent of the calories taken in by

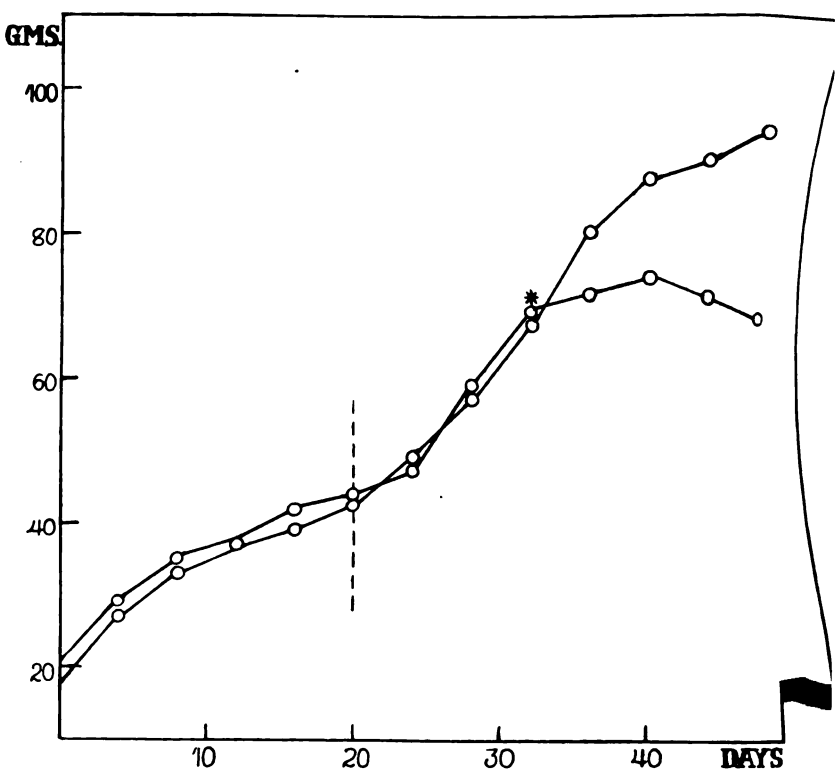

FIG. 3. Upper curve, Rats 33 and 34. Lower curve, Rats 35 and 36. To the left of the dotted line Diet III; to the right, Diet IV. At the point (*) Rats 33 and 34 were changed from Diet IV to Diet II.

imals. Whether yeast alone without butter (replaced by vill produce normal growth in rats, and whether there are more components in yeast which stimulate growth, is investigation.

CALCIUM IN PERMEABILITY AND IRRITABILITY.

By JACQUES LOEB.

(From the Laboratories of the Rockefeller Institute for Medical Research.)

(Received for publication, October 25, 1915.)

I.

There has been a growing tendency to explain all phenomena of
reased activity of the cell on the assumption of an increased
meability. Especially R. S. Lillie[1] has tried to harmonize
ny phenomena with this viewpoint, and it has been adopted
Bayliss[2] in a recent address. Thus it was assumed that the
cies of artificial parthenogenesis induce development by in-
asing the permeability of the egg; that the stimulus of nerve
muscle inducing muscular contraction is due to an increase in
meability induced by the stimulus; that the current of action
gative variation) is due to an increased permeability, and so
Conversely it was assumed that narcosis is due to a diminu-
n in permeability.

The idea that stimulation might be due to an increased per-
ability of the cell originated probably under the influence of
following facts. As is well known, muscular twitchings are
duced when the muscle or the nerve is put into a pure NaCl
tion, while the addition of Ca or Sr or Mg stops these twitch-
s. On the other hand, the writer found that for the egg of
dulus pure NaCl in the concentration in which it occurs in
water is toxic, while the addition of a small quantity of $CaCl_2$
any other salt with a bivalent metal) renders the NaCl harm-
s. In 1905 he suggested that this phenomenon might be ex-
ined on the assumption that in a pure NaCl solution the latter
ers the membrane and kills the egg, while the presence of a
ce of a salt with a bivalent metal prevents this diffusion of NaCl

[1] Lillie, R. S., *Am. Jour. Physiol.*, 1909, xxiv, 14; 1911, xxviii, 197.
[2] Bayliss, W. M., *Science*, 1915, xlii, 509.

into the egg.[3] The correctness of this explanation could later be demonstrated in the following way: When the egg of *Fundulus* is put into a mixture of 50 cc. 3 M NaCl + 1 cc. 10/8 M $CaCl_2$, the embryo will live in this mixture for 3 days or longer, while if the newly hatched fish is put into such a solution it is killed almost instantly. When the egg is put into a pure 3 M NaCl solution the embryo dies within a few hours. These facts seem only intelligible on the assumption that the addition of a trace of $CaCl_2$ to the solution makes the egg impermeable for the NaCl, while without $CaCl_2$ the NaCl gradually diffuses into the egg.

It is, of course, natural to consider the possibility that the stimulating effect of a pure NaCl solution upon nerve or muscle is also due to an increase in permeability, while the $CaCl_2$ inhibits this increase in permeability.

The acceptance of such a view meets, however, with several difficulties. First, there is, for the present, a lack of direct proof for it, and second, it is apparently contradicted by certain facts, one of which may be mentioned. The center of the jellyfish *Polyclonia orchis* will as a rule not contract in an isotonic NaCl solution, but will begin rhythmical contractions when a certain amount of $CaCl_2$ is added; but the contractions can also be called forth if instead of $CaCl_2$ some divalent or trivalent anion is added; *e.g.*, Na_2 tartrate or oxalate or Na_3 citrate.[4] On the basis of our present knowledge it is not probable that small quantities of Ca as well as of oxalate should both increase the permeability of the cell and counteract a diminution of permeability caused by NaCl.

An attempt has been made to connect the electromotive phenomena in living cells with assumed changes in the permeability of the membrane; but these attempts are not warranted.[5]

The writer has recently approached the possible connection of stimulation and permeability from a different viewpoint. Previous experiments had shown that the concentration of $CaCl_2$ (or of salts with bivalent cations) required for the antagonization of salts with univalent cation varies with the concentration of the latter. If the mechanism of antagonization is the same for phe-

[3] Loeb, J., *Arch. f. d. ges. Physiol.*, 1905, cvii, 252.
[4] Loeb, *Jour. Biol. Chem.*, 1905–06, i, 427.
[5] Loeb, *Science*, 1915, xlii, 643.

nomena of irritability as for permeability the ratio of $\frac{C_{Na\ salt}}{C_{Ca\ salt}}$ should vary in the same way for both groups of phenomena with varying C_{Na}. This is not the case.

II.

The eggs of *Fundulus* which normally develop in sea water develop also in distilled water and in solutions of higher osmotic pressure than sea water. If we put the newly fertilized eggs into pure NaCl solutions of different concentrations above 3/8 M NaCl the eggs will form embryos only if a minimal quantity of $CaCl_2$ is added. This quantity varies with the concentration of NaCl. In a series of experiments that quantity of $CaCl_2$ was ascertained which is required to permit 50 per cent of the eggs to form embryos in NaCl solutions of different concentrations. Table I gives the result.

TABLE I.

Concentration of NaCl.	Quantity of M/16 $CaCl_2$ required to allow 50 per cent of the eggs to form embryos.	Concentration of NaCl.	Quantity of M/16 $CaCl_2$ required to allow 50 per cent of the eggs to form embryos.
	cc.		cc.
3/8 M	0.1	9/8 M	1.8–2.0
4/8 M	0.3	10/8 M	2.0–2.5
5/8 M	0.5	11/8 M	2.0(?)*
6/8 M	0.6	12/8 M	3.0–3.5
7/8 M	0.9	13/8 M	6.0
8/8 M	1.2–1.4		

* This value for Ca in an 11/8 M NaCl solution is presumably too low and due to an error.

In NaCl solutions of a concentration beyond 13/8 M it was not possible to cause 50 per cent of the eggs to form embryos, no matter how much Ca was added; in M/4 NaCl, 50 per cent of the eggs could form embryos even without the addition of $CaCl_2$, which might possibly be understood on the assumption that the egg itself contains some $CaCl_2$. This might also explain why so little $CaCl_2$ is needed for the development of the eggs in a 3/8 M NaCl solution.

It is obvious that the minimum amount of $CaCl_2$ which must be added increases much more rapidly than the concentration of NaCl. Thus if the concentration of NaCl varies in the ratio 1:2:3 (if we compare, *e.g.*, 4/8 M, 8/8 M, and 12/8 M NaCl), the values for $CaCl_2$ increase in the ratio of 0.3:1.3:3.2, or, in other words, if we double the concentration of NaCl we must quadruple the amount of Ca added; and if we triple the concentration of NaCl we must add about ten times as much $CaCl_2$. *The value of Ca increases almost in the ratio of the square of the increase of the NaCl solution.*

III.

We will compare with this the variation in the ratio $\frac{C_{Na+K}}{C_{Ca+Mg}}$, *i.e.*, the ratio of the concentration of the chlorides of the monovalent over that of the bivalent cations in the sea water for a case of irritability. As material the newly hatched larvæ of a certain barnacle (*Balanus eburneus*) were used, which can stand wide variations in the concentration of the sea water.[6] These larvæ are strongly heliotropic and gather in dense clusters at the window-side or the opposite side of the dish. They are incessant swimmers and they rise to the surface of the water. They are able to live in sea water from the concentration of M/16 to 6/8 M.

When the larvæ are put into a pure solution of NaCl + KCl (in the proportions in which these two salts exist in the sea water) they will all fall to the bottom, unable to swim, though they may live for a number of hours in such a solution. If one salt with a bivalent cation is added, *e.g.*, $CaCl_2$ or $MgCl_2$ or $SrCl_2$, in sufficient quantity, they will rise to the surface but they cannot stay there very long; if, however, enough of a mixture of $CaCl_2 + MgCl_2$ is added in the proportions in which these two cations exist in the sea water (1.5 atoms of Ca to 11.8 atoms of Mg) the larvæ will rise to the surface and remain there, gathering on the side by the window or away from it.

Experiments were made to ascertain the minimal quantity of $CaCl_2 + MgCl_2$ required to allow all the animals to rise to the surface in different concentrations of NaCl + KCl. Table II gives the results.

[6] Loeb, *Proc. Nat. Acad. Sc.*, 1915, i, 439.

TABLE II.

No. of experiment.	Concentration of NaCl + KCl	Cc. of ½ M $CaCl_2 + MgCl_2$ required.	Value of $\frac{C_{Na+K}}{C_{Mg+Ca}}$
I	M/16	0.3	27.8
	M/8	0.4–0.5	37.0
II	M/8	0.5	33.3
	M/4	0.9–1.0	35.1
III	3/16 M	0.7	35.7
	3/8 M	1.3	38.5
IV	M/8	0.5	33.3
	M/2	1.8–1.9	36.0
V	M/4	0.8–0.9	39.2
	M/2	1.6–1.7	40.3
VI	5/16 M	0.9	46.3
	5/8 M	1.7	49.0
VII	3/16 M	0.6	41.7
	6/8 M	2.4	41.7

Two experiments with concentrations of NaCl + KCl varying in the ratio of 1:2 or 1:4 were always made simultaneously. The permanent readings were taken a number of hours after the animals were put into the solutions. The result indicates that the ratio of $\frac{C_{Na+K}}{C_{Ca+Mg}}$ remains very nearly constant with varying concentrations of C_{Na+K}. This relation corresponds to Weber's law, according to which the change in a stimulus which is just perceptible has a constant ratio to the original stimulus. Weber's law is the most general law in the realm of human sensations and therefore we need not be surprised at meeting such a law in this connection. This side of the problem was discussed in a former paper.[7]

It is, therefore, obvious that the ratio of $\frac{C_{Na}}{C_{Ca}}$ for the phenomenon of irritability selected for discussion varies according to a different law than for the case of permeability. Our results, therefore, do not lend support to the idea that the rôle of calcium in phenomena of irritability is the same as in phenomena of permeability.

[7] Loeb, *Proc. Nat. Acad. Sc.*, 1915, i, 439.

IV.

Not only in NaCl + KCl but also in NaCl + KCl + $MgCl_2$ are the larvæ unable to rise for any length of time to the surface, while if we add some Ca the larvæ will do so. Experiments of the following kind were made. To 50 cc. M/2 NaCl + KCl were added different quantities of $MgCl_2$, and it was ascertained how the quantity of $CaCl_2$ necessary to cause the larvæ to rise and remain at the surface varied with the amount of Mg added. In former investigations the writer had shown that the swimming motions of the center of a jellyfish cannot continue in a mixture of Na + K + Mg, but that this effect of Mg can be promptly overcome by the addition of Ca;[8] and this antagonism between Ca and Mg was confirmed by Meltzer and Auer[9] in their experiments on mammals.

Our experiments consisted in adding to 50 cc. M/4 or M/2 NaCl + KCl (in the proportions in which these salts exist in sea water) varying quantities of 3/8 M $MgCl_2$. In such solutions the animals could swim for only a few minutes. If, however, some $CaCl_2$ was added the animals could rise permanently to the surface and swim to or from the window-side of the dish. It was ascertained how much $CaCl_2$ was required to cause the majority of the larvæ to rise. Table III gives the results.

TABLE III.

	Cc. of M/16 $CaCl_2$ necessary to induce the majority of the larvæ to swim in:	
	M/2 Na + K	M/4 Na + K
50 cc. NaCl + KCl + 0.75 cc. 3/8 M $MgCl_2$....		0.2
" 1.5 "	0.4	0.3
" 2.5 "	0.4	0.4
" 5.0 "	0.7–0.8	0.7–0.8
" 10.0 "	1.6	1.6
" 15.0 "	1.8	
" 20.0 "	1.8	

[8] Loeb, *Jour. Biol. Chem.*, 1905–06, i, 427.

[9] Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1908, xxi, 400.

In order to interpret these figures correctly we must remind the reader that we are dealing here with a combination of two antagonisms. The one is between the salts with univalent and bivalent metals. This antagonism is satisfied by merely adding enough $MgCl_2$ to a mixture of NaCl + KCl. The reader will recall that in a mixture of NaCl + KCl + $MgCl_2$ the larvæ will swim for a few minutes if enough $MgCl_2$ is added. The second antagonism is between $CaCl_2$ and $MgCl_2$. With the addition of only $MgCl_2$ the animals can swim but a short time; but if both $MgCl_2$ and $CaCl_2$ are added in the right concentration all the larvæ will swim permanently.

In the experiments in Table III enough $MgCl_2$ was always present (with the exception of the first solution) so that the balance between salts with univalent and bivalent cations was established. What was lacking was the balance between Ca and Mg. The experiments of Table III therefore answer the question of how the concentration of Ca must change if the concentration of Mg changes. If we consider only the concentrations of Mg between 2.5 and 10.0 cc. 3/8 M Mg, we find again that the C_{Ca} must vary directly in proportion to C_{Mg}, which again is Weber's law. Thus if the $MgCl_2$ added varies from 2.5: 5: 10.0 cc., *i.e.*, in the ratio of 1: 2: 4, the quantities of $CaCl_2$ required are 0.4: 0.8: 1.6 cc., which is also the ratio of 1: 2: 4.

The normal concentration of Mg is about 6.0 cc. in 50 cc. of solution. Hence, as long as the concentration of Mg is neither excessively high nor low, the law of proportion nearly holds. Only when the concentration of Mg is very low or excessively high do we find deviations from this law; but this is a peculiarity which we find in all other cases of Weber's law.

It agrees also with our statement that it makes no difference whether the 50 cc. NaCl + KCl are present in M/4 or M/2 solutions, since we are dealing here only with the antagonism between Ca and Mg.

Experiments in which the original mixture was NaCl + KCl + $CaCl_2$, and where the quantity of $MgCl_2$ required to induce most or all of the larvæ to swim was ascertained, gave no results which could be utilized for quantitative measurements, for the reason that it is impossible to find a sharp end-point which could serve as a standard of measurements. In a mixture of NaCl +

KCl + $CaCl_2$, when the concentration of $CaCl_2$ is normal or l[illegible]e
low normal, the larvæ lie a long time on the bottom of the di[illegible]sh
finally some will rise and swim to the light. The number wh[illegible]cl
will swim will be increased by the addition of $MgCl_2$, but not in
a way which permits quantitative experiments.

SUMMARY.

The variation of the amount of Ca, or of Ca + Mg, requi[illegible]ed to antagonize various concentrations of NaCl, or of NaCl + K[illegible]Cl, was investigated for a case where the antagonism concerned t[illegible]he permeability, and for a case where it concerned irritability. It was found that in the case of irritability the Ca required varied in direct proportion to the change in the concentration of NaCl (Weber's law), while in the case of permeability the concentration of Ca required for antagonism varied approximately with the square of the ratio of the concentration of NaCl.

THE SALTS REQUIRED FOR THE DEVELOPMENT OF INSECTS.

By JACQUES LOEB.

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I.

The writer reported recently that it is possible to raise the banana fly on a sterilized liquid medium consisting of water, one or two sugars (grape sugar and cane sugar), one ammonium salt (*e.g.*, ammonium tartrate), and some inorganic salts.[1] Such a mixture is a well known culture medium for certain microorganisms; *e.g.*, yeast cells, which are capable of synthetizing their proteins and other complicated organic compounds from ammonium salts. He left the question undecided whether or not microorganisms (either in symbiosis with the fly or carried with it to the culture medium) acted as an intermediate in this synthesis, and he is not yet ready to give an answer. He has since tried to find out which inorganic salts are required for the completion of the life cycle of the fly. This question is interesting for the following reason. For microorganisms the indispensable ions are, as a rule, K, Mg, PO_4, and SO_4, while very often neither NaCl nor $CaCl_2$ is required. In 1900 the writer called attention to the fact that for the rhythmical contractions of the jellyfish, *Gonionemus*, NaCl was required and that for the continuation of these contractions NaCl and $CaCl_2$ were required in certain proportions.[2] Lingle confirmed this for the heart beat of the tortoise,[3] and Overton showed later that the nerve and muscle of the frog lose their irritability reversibly if they are kept for some time in a sugar solution, while their irritability is preserved if a slight amount of NaCl is added to the sugar solution.

[1] Loeb, J., *Science*, 1915, xli, 169.

[2] Loeb, *Am. Jour. Physiol.*, 1900, iii, 383.

[3] Lingle, D. J., *Am. Jour. Physiol.*, 1902-03, viii, 75.

The banana fly possesses a high degree of motility, and it was therefore of interest to know which inorganic salts would be required to raise a number of successive generations possessing normal activity.

The experiments were carried on in a platinum vessel. The nutritive solutions consisted of the following mixture:

	gm.
Grape sugar	0.5
Cane sugar	0.5
Ammonium tartrate	0.1
Citric acid[4]	0.05
K_2HPO_4	0.005
$MgSO_4$	0.005
H_2O	3 cc.

All the substances used were the purest that could be obtained.

The solution was put into a platinum vessel. Into this vessel was put a basket of silver netting which just touched the upper surface of the solution. The flies were put into this basket, which allowed them to lay the eggs on the surface of the nutritive solution but prevented the flies from falling into the liquid and drowning. The platinum vessel was put into a glass cylinder about 10 cm. high which was closed with absorbent cotton. Before the beginning of the experiment the whole was sterilized by heating in an autoclave to 120° for one hour. At first three pairs of flies were put into the vessel, left there for four days in order to lay their eggs, and then quickly removed. After this the flies raised in the platinum dish were used for propagation in the manner described.

Thus far, five successive generations of flies have been raised under these conditions in the platinum vessel. The motility of the flies is perfectly normal. The experiments show that without any other NaCl or $CaCl_2$ than that which may appear as impurities in the chemicals used, five and probably indefinite generations of flies can be raised.

The only salts added were K_2HPO_4 and $MgSO_4$. Numerous control experiments made in glass vessels showed that without either the addition of K or PO_4 no larvæ can be raised. When

[4] The citric acid was added to keep the solution acid and to exclude the development of bacteria as much as possible.

Na was substituted for K no flies could be raised. I am not sure whether Mg and SO_4 are as indispensable as K and PO_4, since in K_2HPO_4 alone occasionally a fly developed. It is certain, however, that the addition of $MgSO_4$ greatly increased the number of flies raised.

As far as the evidence from these experiments goes we can, therefore, say that in these flies the muscular activity is possible either without any Na or Ca or with only such traces as appear in the form of impurities in the chemically pure substances used in these experiments; while K as well as PO_4, and also SO_4, and Mg must be added to the culture medium in appreciable quantity.

We intend to repeat these experiments with substances which shall be absolutely free from Na and Ca.

II.

The experiments show that as highly organized an animal as the banana fly can be raised on a culture medium as simple as that required for certain microorganisms.

As far as the writer is aware it is generally assumed that the evolution of higher animals could only have taken place after green plants had come into existence, since the latter serve directly or indirectly as food for the animals. While this is generally true for our present fauna, the possibility is not excluded that an evolution of animals as highly specialized as insects might have taken place independently of the existence of green plants.

The investigations of Winogradski[5] and of Godlewski on nitrite and nitrate bacteria seem to have made it certain that these organisms are capable of forming carbohydrates from carbon dioxide (or possibly other carbon compounds in the air) independently of light; and the same may be true for certain other microorganisms. Microorganisms of this type might, therefore, suffice to furnish the carbohydrates necessary for the development of other microorganisms which require sugars for their growth. Even if we assume that in our experiments yeast cells[6] or other microor-

[5] Winogradski, S., *Handb. d. tech. Mykol.*, 1904, iii, 162. See also Beijerinck, M. W., *Folia Microbiologica*, 1914, iii, 91.

[6] Guyénot, E., (*Compt. rend. Soc. de biol.*, 1913, lxv, pt. i, 178) has shown that yeast can serve as food for *Drosophila*, and it has been stated that in Germany yeast has become a general food for higher animals.

ganisms acted as intermediates in the building up of proteins for the fly (which is quite possible), it is obvious that an evolution of animals as complicated as the banana fly (which usually lives on plant food) might have been possible without the existence of chlorophyll, provided that Winogradski's conclusions are correct.

THE OCCURRENCE OF PITUITRIN AND EPINEPHRIN IN FETAL PITUITARY AND SUPRARENAL GLANDS.

By CAREY PRATT McCORD.

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(Received for publication, October 4, 1915.)

For the growth of the fetus *in utero* it is undetermined whether the greater influence is exerted by the maternal internal secretory system or by the newly formed glands of the fetus itself. The added strain incident to pregnancy is evidenced in the mother's glandular system by such functional hyperplasias as the frequently observed hyperthyroidism or the altered facies indicative of a mild, transient acromegaly from hyperpituitarism. At what period of development the embryo comes under the influence of the secretions of its own glands is not known. An investigation to establish the earliest developmental period in which it is possible to detect the presence of the glandular secretions in the glands themselves would be of value to the full solution of this problem. The work which led to this report was the examination of the pituitary and suprarenal glands of bovine fetuses, from full term back as early as the macroscopic recognition of the glands was possible, in an effort to establish the stage at which these glands commence the elaboration of their active principles.

Forty-two embryos in various developmental phases were procured in fresh condition from the abattoirs. In those that were at or near full term, no difficulty was encountered in the separation of the anterior and posterior lobes of the pituitaries. In younger embryos separation was not possible and the entire gland was tested. In the youngest fetuses it was necessary to freeze the bodies to facilitate the removal of the glands. To provide sufficient material to bring about the characteristic physiologic reactions, several of the youngest embryos of approximately the same ages were grouped and tested as one. In every instance,

the material was extracted with distilled water and the extract freed of protein contamination.

The presence of the active principle of the pituitary was measured in terms of oxytocic activity, by means of the method of Dale and Laidlaw[1] with histamine (β-imidazolyl ethylamine) as a standard (Roth[2]). This oxytocic test under optimum working conditions has proven qualitatively active with special pitu tary preparations in dilutions 1 to 1,000,000,000. Since these especially prepared pituitary preparations are known to be five times as active as histamine, the statement above is grossly in accord with that of Roth that the test is sensitive to 1 part of histamine in 250,000,000. As a method of quantitative assaying, this oxytocic test requires the most exact technique. With proper consideration for the many apparently trivial causes of error, exquisitely accurate results may at times be obtained. As a routine procedure for the accurate standardization of a large number of preparations, the method is not attended with such ease of manipulation as is suggested in some of the published articles descriptive of this test.

The presence or absence of epinephrin in the adrenal glands was detected through observations of the influence of the several extracts in relaxing the contracted uterine muscles of rodents. The tests were made upon such guinea pig uterine muscles as were refractory in that there occurred no ready spontaneous relaxation after being stimulated to contraction by histamine. Earlier Fenger[3] examined the suprarenals of fetuses for the presence of the crystalline epinephrin. He was able to recover epinephrin in all fetuses examined, but his studies did not include the early weeks of development. This writer also points out the occurrence of iodine in fetal thyroids.

The tests for pituitrin were begun with the embryos at or near full term. Such tests and others back as early as nine weeks were quantitative tests. Pituitrin was present in all extracts, and the quantity for a unit of weight was larger than for the adult. The quantity present in the several stages examined was in proportion to the stage of development. At a period represented by the

[1] Dale, H. H., and Laidlaw, P. P., *Jour. Pharm. and Exper. Therap.*, 1912–13, iv, 75.

[2] Roth, G. B., *Bull. Hyg. Lab., U.S.P.H. and M.-H.S.*, No. 100, 1914.

[3] Fenger, F., *Jour. Biol. Chem.*, 1912, xii, 55.

seventh and eighth weeks, the contents of the cranium were grossly only a viscid mass in which the pituitary could no longer be recognized although the sella turcica was plainly visible. Physiologic testing of the pituitary was not feasible for this or any earlier stage. It may be recalled that at this approximate stage the developing anterior lobe encloses and invades the *pars nervosa* with a layer of cells that later becomes the *pars intermedia*, which probably is the actual secreting portion of the posterior lobe. Thus the testing for pituitrin is positive at a time which approximated the earliest period when on theoretical grounds secretion is at all probable.

The parallel testing of the adrenal extracts for physiologic evidence of the presence of epinephrin indicates that epinephrin was present at all stages examined. Even when the pituitaries were no longer obtainable in the very young embryos, the adrenals were distinct entities and readily obtainable. At the end of the sixth week, the epinephrin tests were distinctly positive.

Further details as to weights, ages, etc., are grouped in the table that follows.

SUMMARY.

Physiologic reactions characteristic of extracts of pituitary and suprarenal glands have been obtained from bovine fetal glands during all developmental stages in which the macroscopic recognition of the glands is possible. For the pituitary gland, this period is from the eighth week to full term; for the suprarenals the period is from the sixth week to full term. The presence of the active principles of these glands at so early a developmental period suggests that the fetus *in utero* may be under the influence of its own internal secreting glands as well as the maternal glands.

The Occurrence of Pituitrin and Epinephrin in Fetal Glands.

Group.	No.	Length of fetus.	Approximate age.	Weight of pituitary.		Weight of suprarenal (both glands).	Oxytocic testing.		Remarks.
				Whole.	Posterior lobe.		Pituitary.	Suprarenal.	
		mm.	*days*	*gm.*	*gm.*	*gm.*			
I	1	113	55			0.0345	Pituitary not recognizable	Active	The 5 embryos were grouped and tested as one.
	2	127	59						
	3	130	60						
	4	140	63			0.0336			
	5	152	65						
II	6	165	67			0.0764	Active	Active	Nos. 6, 9, and 11 were grouped together to be tested. Extract made from the pituitary glands removed from Nos. 7, 8, 10, and 12 was tested quantitatively.
	7	♀ 175	68	0.0032	0.0016	0.099	"	"	
	8	♀ 187	69	0.015	0.0048	0.087	"	"	
	9	201	70	0.0214		0.145	"	"	
	10	♀ 213	72	0.0105	0.0035	0.09	"	"	
	11	218	73	0.023		0.156	"	"	
	12	♀ 220	74	0.0165	0.006	0.109	"	"	
III	13	♀ 223	75	0.0185	0.0066	0.113	"	"	Nos. 14, 15, 16, and 17 were grouped together to be tested. No. 13 was tested quantitatively.
	14	225	75	0.0278		0.0824	"	"	
	15	235	75	0.0238		0.102	"	"	
	16	240	76	0.0325		0.102	"	"	
	17	245	76			0.161	"	"	
IV	18	250	77	0.034		0.160	"	"	Nos. 18, 19, and 20 were grouped together to be tested.
	19	250	77	0.03		0.134	"	"	
	20	265	79			0.15	"	"	
V	21	270	80	0.0922		0.222		"	Nos. 21, 22, and 24 were grouped together and tested. The extract made from the pituitary glands removed from Nos. 23, 25, 26, and 27 was tested quantitatively.
	22	275	81			0.171		"	
	23	♂ 280	82	0.026	0.009	0.180	"	"	
	24	290	83	0.0224		0.122		"	
	25	♂ 298	84	0.032	0.0085	0.139	"	"	
	26	♀ 300	140	0.0415	0.0065	0.209	"	"	
	27	♀ 320	150	0.04	0.0105	0.268	"	"	

THE RESUMPTION OF GROWTH AFTER LONG CONTINUED FAILURE TO GROW.[1]

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

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(Received for publication, October 22, 1915.)

The failure of an individual to grow or to complete its growth in a normal manner during the period in which this function ordinarily is exercised raises a number of problems of far reaching physiological importance. Some of these have been considered in detail in an earlier paper.[2] Contrary to the belief expressed by a number of prominent physiological writers, it was demonstrated that even if growth is repressed for a long time the capacity to grow is not necessarily lost at the end of the period at which growth ordinarily ceases in any species. For example, we presented the record of an albino rat (1012♂) which had not exceeded a body weight of 127 gm. at the end of 370 days of age. This is approximately 100 days beyond the age at which growth ordinarily ceases and is well beyond the middle of life in individuals of rat colonies maintained under our laboratory conditions. Nevertheless on an appropriate dietary this animal promptly began to grow again, reached a satisfactory maximum weight of 280 gm. by growing at a rate approximately normal for its size, and continued to live until the age of about 700 days when it died of lung disease.

The foregoing instance of the capacity of a dwarfed individual to grow at an age where others of the same species have, by

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[2] Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1914, xviii, 95.

growing, lost the ability to undergo further increment in size is not an isolated case. Few investigators before us have succeeded in repressing growth in this manner beyond the period of adolescence. The earlier trials of Aron, who likewise found a capacity to grow long retained, were not thus prolonged. In the case of dogs he believed that it was ultimately lost when an age corresponding to adult life was reached.[3] Subsequently Aron's experience with rats led to a different statement. Thus he wrote:

Die Wachstumsfähigkeit geht also auch durch langdauernde Perioden des Wachstumsstillstandes nicht verloren; der Wachstumstrieb bleibt über die Dauer der Wachstumshemmung hinaus erhalten, unter Umständen sogar bis in ein Alter hinein, in dem sich normalerweise die Wachstumsfähigkeit schon zu verlieren beginnt oder gänzlich erloschen ist. Diese Feststellung, dass der Wachstumstrieb, jene den Keimzellen immanente, von ihr auf alle anderen Zellen des jugendlichen Organismus vererbte Kraft nach langdauernden Wachstumshemmungen infolge Unterernährung latent bleiben kann, dass ein im Wachstum gehemmtes Tier noch in einem Alter zu wachsen vermag, in dem seine normalen Geschwistertiere diese Fähigkeit physiologisch verloren haben, ist deshalb wichtig, weil man bisher immer von der Voraussetzung ausgegangen ist, dass Wachstumsfähigkeit und Jugend in einem ursächlichen Zusammenhang stehen, dass der Wachstumstrieb als eine spezifische Eigenschaft der jugendlichen Zellen anzusehen ist. Da nach unseren Versuchen Wachstumsfähigkeit und Intensität des Wachstumstriebes nicht mehr als eine Funktion des Lebensalter gelten können, werden auch alle jene Theorien hinfällig, welche den Ablauf des Wachstumsvorganges abhängig machen wollen von der nach der Befruchtung der Eizelle verstrichenen Zeitspanne, von der Zahl deim Körper abgelaufenen Zellteilungen oder, wie Rubner annimmt, von deGrösse der umgesetzten Calorienzahl. Ob sich bei noch länger fortgesetzter Wachstumshemmung nicht schliesslich ein Zeitpunkt im Leben der Tiere erreichen lässt, an dem die Wachstumsfähigkeit völlig erlischt, darr auf bleiben meine Versuche noch die Antwort schuldig; begnügen wir unmit der Feststellung, dass ein durch Unterernährung im Wachstum ges

[3] Aron, H., Weitere Untersuchungen über die Beeinflussung des Wachstums durch die Ernährung, *Verhandl. d. 29sten Versammlung d. Gesellsch. f. Kinderh.*,1912, p. 99, wrote of these experiments: "Die Versuche ergeben ohne Zweifel, dass die Tiere trotz der intensiven Wachstumshemmung, die sich aber nicht über die ganze Jugendzeit erstreckte, noch über eine lebhafte Wachstumsfähigkeit verfügen. Recht interessant ist im Gegensatz hierzu das Ergebnis eines früher angestellten Hundeversuches, der darauf schliessen lässt, dass die Wachstumsfähigkeit erlischt, wenn die Nahrungsbeschränkung und der Wachstumsstillstand sich über die ganze Jugendzeit ausdehnen."

hemmtes Tier seine Wachstumsfähigkeit bis in ein Alter hinein behält, in dem diese normalerweise schon erloschen ist.[4]

We have already observed the resumption of growth in several rats after its suppression for more than 500 days—twice the age at which adequate size is ordinarily reached by the normally developing individuals in the same environment. A partial record of one of them was presented in our previous paper,[5] Chart II, 531 ♀. Some of the statistics in relation to animals stunted more than 400 days are given below. Animals of different sizes during the stunting period are represented.

Capacity of Albino Rats to Grow at Very Late Age after Suppression of Growth.

Rat.	Growth resumed at		Final maximum body weight.
	Age.	Body weight.	
	days	*gm.*	*gm.*
531 ♀	552	170	204
2031 ♀	537	108	187
2033 ♀	512	58	222
569 ♀	479	167	228
2339 ♀	401	104	259

It should be noted in connection with the foregoing individuals that their curve of growth after the period of suppression was as a rule comparable with that of a growing rat of the same size and sex. The usual rate of body increment was not diminished, but, if anything, was sometimes somewhat accelerated during the resumption of the growth function.[6] How much longer than 552 days the capacity to grow can be retained in albino rats remains

[4] Aron, H., Untersuchungen über die Beeinflussung des Wachstums durch die Ernährung, *Berl. klin. Wchnschr.*, 1914, li, 972.

[5] Osborne and Mendel, The Suppression of Growth and the Capacity to Grow, *Jour. Biol. Chem.*, 1914, xviii, 105.

[6] The data for two male rats, 2036 and 2038, in which growth was suppressed until the ages of 532 and 509 days respectively are not included in the tabular summary above. They resumed growth promptly when a suitable diet was offered, and grew from 108 to 180 gm. and from 122 to 230 gm. respectively. An accident in the laboratory terminated these experiments before they were carried to a satisfactory conclusion.

to be ascertained; at any rate there was no sign of an incipient impairment in the experiments recorded. To enable the reader to appreciate the full significance of the data presented we may remark that fully half of our stock rats have died before the age of 600 days.

Among the inquiries raised in relation to the resumption of growth is the question as to whether, despite the demonstrated renewal of growth, animals that have long been stunted can ever reach the *full* size and physical equipment characteristic of unretarded individuals. According to Aron, who has also considered this feature, prolonged stunting of rats through the usual period of growth leads to permanent damage. Thus he writes:

> Eine andere Frage ist nun, ob durch intensive und langdauernde Unterernährung im Wachstum gehemmte Tiere imstande sind, das Versäumte im höheren Alter restlos wieder nachzuholen und normale Grösse und normales Gewicht zu erreichen. Erstreckt sich die Wachstumshemmung nicht über eine allzu lange Zeitspanne (etwa 50–150 Tage bei Ratten), so tritt wohl eine zeitliche Verzögerung im Ablauf des Wachstumsvorganges ein, die Tiere erreichen schliesslich in entsprechend höherem Alter aber ein Gewicht und eine Grösse, die jedenfalls nicht nennenswert von der der normal ernährten Geschwistertiere abweicht. Wurden die Tiere aber im Wachstum solange zurückgehalten, bis die normalen Vergleichstiere völlig ausgewachsen erschienen, und jetzt erst aufgefüttert, so wachsen sie zwar noch, erreichen endgültig aber nicht mehr Gewicht und Grösse eines normalen Tieres. Derartig langdauernde Wachstumshemmungen haben also eine dauernde Schädigung zur Folge.[4]

A detrimental effect of the sort here described is by no means a necessary outcome of prolonged stunting of rats by means of dietary deficiencies. We have selected for the appended chart (Rats 1012, 2031, 2033, 2161, 2180, 2339, 2476) a number of illustrations of attainment of adequate body weight after suppression of growth during a period essentially equal to or exceeding the normal growth period. The preliminary failure to grow in the cases here selected from typical experiments was brought about by a variety of intentionally enforced dietary conditions, such as limited quantity of food (2033 ♀), rations low in protein (2180 ♂, 2339 ♀), qualitatively inadequate proteins (2031 ♀, 2161 ♂, 2476 ♀), and the use of artificial "protein-free milk" in the diet (1012 ♂).

Many of the attempts, after suppression of growth, to bring about as perfect a completion of the developmental processes as is indicated in the preceding discussion have failed. The question has been raised as to whether the initial age or size at which the stunting began was of consequence. A *brief* period of suppressed growth at any age is without detriment. It is easily conceivable that long continued dwarfing at a period of development represented by, say, 60 gm. body weight might be far more damaging than an equally long suppression at 160 gm. or some period nearer adult size. If the growth impulse decreases, as some believe, with increasing growth and age, it may be greater in a small rat than in one two-thirds grown, even after long periods of suppression. Our records show numerous *prompt responses to the opportunity to complete growth at all sizes, after suitable types of suppression of growth.* The sexual maturity of rats is reached somewhere about the ages at which the body weight here represented is normal. This too may be a potent factor. We have therefore supplemented our earlier experiments by stunting trials in which the retardation of growth was brought about at comparatively early age. The series of explanatory data tabulated below, part of which are reproduced graphically in the appended chart, show that *even when the stunting is attempted for very long periods from an early age the capacity to resume growth adequately is retained.*

Whether animals which resume their growth at a very late age develop into individuals normal in every respect besides their external appearance, rate of growth, and ultimate size, is not so easily answered. It is conceivable that during the stunting period certain tissues are permanently impaired without furnishing evidence by any of the criteria which we have selected. It is also not impossible that developmental changes may proceed even in the absence of an increment of size. A true "dwarf" may exhibit the form and proportions of an adult while retaining a very small size. Aron has cited the case of a young rat which had been kept at the constant weight of 56 gm. during 50 days and yielded a total ash of 5.05 gm. in contrast to a content of 2.1 gm. ash in a growing rat weighing 62 gm. This more than twofold increment during stationary weight suggests a continuance of osseous changes even in the absence of growth of the body as a whole.

*Summary of Experiments on Resumption of Growth after Prolonged Periods of Suppression of Growth.**

Rat.	Stunting began at		Growth resumed at		Maximum body weight attained after growth was resumed.
	Body weight.	Age.	Body weight.	Age.	
	gm.	*days*	*gm.*	*days*	*gm.*
1892 ♀	50	35	51	220	192
2033 ♀ †, ‡	53	39	59	513	222
2028 ♀ ‡	53	44	60	329	185
2154 ♀	59	43	65	254	183
2293 ♂	68	49	69	193	414
2161 ♂ †, ‡	53	38	73	248	309
240 ♀	34	38	73	314	159
2180 ♂ †	52	35	73	303	376
2362 ♀	74	45	76	154	165
1150 ♀	55	33	92	151	197
2342 ♂	92	57	92	148	276
2461 ♀	92	42	92	185	173
2435 ♀	90	48	96	337	162
2463 ♀	96	42	97	231	217
2343 ♂	98	57	98	190	306
2116 ♀	60	34	103	311	195
1123 ♀	96	55	119	215	189
2339 ♀ †	48	39	104	401	259
2369 ♀	79	45	104	380	247
2114 ♀	65	42	106	321	202
1113 ♀	95	56	107	237	174
1109 ♀	64	45	108	166	169
2031 ♀ †, ‡	48	44	108	537	188
2036 ♂	52	39	108	532	180
1213 ♀	56	31	109	169	171
2104 ♀	89	44	109	322	282
2126 ♀	87	53	109	158	164
1568 ♂	52	30	117	157	292
2476 ♀ †	54	31	118	322	231
1696 ♀	68	43	122	271	170
2038 ♂	46	37	122	509	232
1012 ♂ †	69	47	127	371	281

* It should be remembered in considering these data that the average maximum reached by our rats is: for females, about 200 gm.; males, about 300 gm. Many of the experiments were undertaken for different purposes than those here indicated and were stopped before the maximum possible weight was attained.

† The illustrative growth records of these rats are shown in the appended chart.

‡ Photographs of this rat before and after resumption of growth are reproduced in the text.

on has emphasized changes of form undergone by animals which
e maintained at stationary weight before the completion of
owth—a feature which Waters has pointed out in his studies
the capacity of cattle to grow under adverse conditions.[7] Ac-

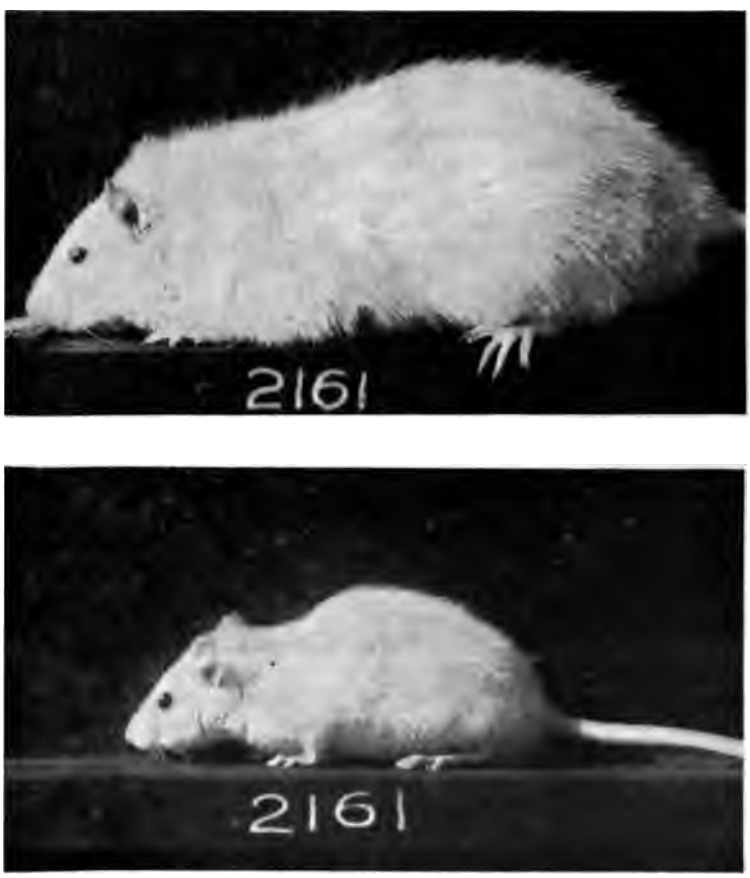


FIG. 1. Rat 2161♂ (below) photographed at the age of 246 days, and
ly weight of 71.5 gm. The diet contained zein with very small addi-
ns of tryptophane and lysine. The upper picture shows the animal
ter resumption of growth) at the age of 459 days and body weight of
gm.

[7] This topic is discussed in our monograph, *Carnegie Institution of Wash-
ton, Publication No. 156*, pt. ii, 1911.

cording to Aron the alterations in external form that he has observed during cessation of growth are not as conspicuous in rats as in dogs which were the subjects of his earliest investigations. We have not been impressed by the appearance of such distortions of form in our rats during prolonged stunting, but as yet we have no actual measurements upon which the decision as to the occurrence of such phenomena must actually be based. Inasmuch as they are supposed to involve the skeletal parts, it ought not to be difficult to get convincing data.

The comparable photographs[8] of Rat 2161 ♂ show the animal (below) at the age of 246 days and 71.5 gm. body weight after

FIG. 2. Rat 2028 ♀ photographed at the age of 325 days and at a body weight of 55 gm., after 281 days of stunting on a limited daily quantity of food. This animal subsequently grew to the usual adult size of 185 gm. on a suitable diet.

a prolonged suppression of growth on a diet in which the essential nitrogenous component consisted of the protein zein with the addition of tryptophane and lysine in small amounts (see page 449). Growth was resumed at the age of 248 days on a diet in which zein and the amino-acids were replaced by casein. The upper photograph shows the same rat at the age of 459 days with

[8] All of the photographs reproduced in the text were made under exactly comparable conditions with respect to focal distance, apparatus, etc.

a body weight of 295 gm. The growth curve is plotted in the appended chart. Rat 2028 ♀ is shown stunted at the age of 325 days and body weight of 55 gm. This animal was fed during 281 days on a quantity of milk food so limited daily that no increment in weight was possible. It subsequently resumed growth satis-

FIG. 3. Rat 2033 ♀ (below) photographed at an age of 505 days after prolonged stunting at a body weight of 53 gm. Growth was prevented by limiting the daily quantity of food. The upper photograph shows the animal after resumption of growth on a suitable food to a body weight of 205 gm.

factorily when the ration was made more liberal, reaching a body weight of 185 gm. Rat 2033 ♀ (below) was similarly stunted to 505 days of age, at a body weight of 53 gm. Rat 2031 ♀ (below) was stunted to 510 days and a body weight of 102 gm. on a diet in which the protein was derived from corn gluten. Both

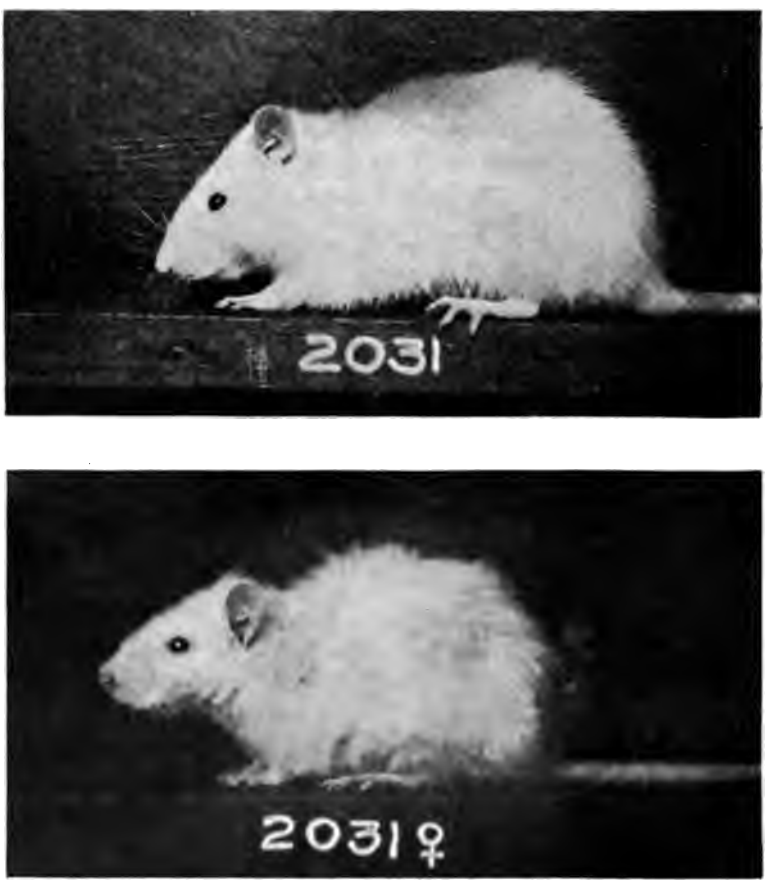


FIG. 4. Rat 2031 ♀ (below) photographed at an age of 510 days, after slow growth, to a body weight of 102 gm., was made on a diet in which the protein was derived from corn gluten.

The upper photograph shows the animal after resumption of growth on a suitable food to a body weight of 188 gm.

of these rats resumed growth on suitable rations and reached body weights as follows: 2033 ♀, 205 gm.; 2031 ♀, 188 gm., when they were photographed as shown in the upper pictures in each case.

From our own experience we can at least say that *the procreative functions are not necessarily impaired by stunting before the age at which breeding is ordinarily possible.* We have kept two rats, 2154 ♀ and 2161 ♂,[9] at body weights not exceeding 65 and 73 gm. respectively from the ages of 43 and 38 days until they were 253 and 248 days old. The failure to grow was due to the qualitative insufficiency of the protein of the diet. It consisted of zein with the addition of the amino-acids tryptophane and lysine in small amounts—just sufficient for maintenance without growth. The composition of the food was essentially as follows:

	per cent
Protein and amino-acids	18
Starch	27
Lard	9
Butter fat	18
"Protein-free milk"	28

Growth was resumed when the nitrogenous components of this diet were replaced by casein or lactalbumin. The animals were subsequently paired and the female gave birth to eight young when she had reached an age of 310 days. Other rats stunted for long periods of time have likewise given birth to young when the resumption of growth was completed. For example Rat 2339 ♀ and Rat 2369 ♀ (see table, page 444) gave birth to young at the ages of 535 and 510 days respectively. No damage to the maternal functions from the suppression of growth was here manifested.

There are, of course, many ways of inhibiting growth. Insufficient food, insufficient protein or inorganic salts, or unsuitable protein each may be the contributory cause. It does not follow that the outcome of the suppression will be equally harmless in every case. Among the many records of experiments which we have conducted with various objects in view, other than those

[9] See appended chart for the growth curve of this rat, of which photographs are shown in the text.

specially considered in this paper, there are a number in which animals were maintained at stationary weight or experienced unusually slow growth owing to *special* deficiencies of their diet. By decreasing the content of protein alone in a ration which unchanged would in every way suffice for adequate growth, it becomes possible to decrease the increment of body weight at will. With lower and lower proportions of protein growth becomes correspondingly less rapid. In this way the content of protein can be reduced to a level where no growth whatever results, but the animals are maintained at constant body weight.[10] Receiving an abundance of all the nutrients except protein, they may be kept stunted on the low protein ration for long periods of time.

Aron looks upon the suppression of growth by furnishing a ration low in protein alone as more deleterious for a subsequent renewal of growth than is underfeeding with a food mixture entirely adequate, if fed liberally, for normal growth.

Auch nach einer durch fortgesetzte eiweissarme Ernährung hervorgerufenen Wachstumshemmung, durchgeführt, bis die eiweissreich ernährten Geschwistertiere ausgewachsen erscheinen und Junge gezeugt haben, beginnt bei Uebergang auf die normale, eiweissreiche Ernährung, das bis dahin zurückgehaltene Gewichts- und Grössenwachstum sich aufs neue zu entfalten. Eine längere Periode eiweissarmer Ernährung scheint aber den Wachstumstrieb deletärer zu beeinflussen als eine im übrigen ähnliche Wachstumshemmung durch Unterernährung; die Tiere wachsen zwar noch, erreichen aber auch bei fortgesetzt eiweissreicher Ernährung im höheren Alter nicht mehr die Maasse eines von vornherein eiweissreich gefütterten Kontrolltieres. Meinen bisherigen Versuchen an Ratten glaube ich entnehmen zu müssen, dass eiweissarme Ernährung in der Jugendzeit die Tiere nachhaltiger schädigt als einfache Unterernährung.[4]

As an illustration Aron shows the growth curves of rats growing on a diet containing 12 per cent of casein, and of others stunted by the same food containing only 2.5 per cent of the protein. The latter animals started to grow when more adequate food was furnished to them at the age of about 190 days; but they failed to reach full adult size.

The records of a not inconsiderable number of our own animals which were prevented from growing for long periods by the deficiency of protein in the ration, and were subsequently fed a

[10] Compare Osborne and Mendel, *Jour. Biol. Chem.*, 1915, xx, 351.

more suitable food mixture, show that the foregoing statement of Aron is by no means tenable as a general rule. A few illustrative records are summarized in tabular form.

*Tabular Summary of Growth Records of Rats after Early Prolonged Suppression of Growth Owing to Low Concentration of Protein in the Food Mixtures.**

Rat.	Duration of restricted diet.	Age at which normal growth was resumed.	Maximum body weight during stunting periods.	Maximum body weight finally reached.	Age at time of maximum weight.
	days	*days*	*gm.*	*gm.*	*days*
2049 ♀ †...				184	413
2051 ♂ †...				327	549
2104 ♀....	279	322	109	276	580
2112 ♀....	267	309	108	206	514
2113 ♀....	278	320	127	224	524
2114 ♀....	280	322	106	202	367
2116 ♀....	277	311	103	195	446
2180 ♂....	278	303	73	376	487
2293 ♂....	155	193	69	434	497
2321 ♀....	98	144	74	172‡	229
2342 ♂....	91	148	92	276‡	333
2343 ♂....	133	190	98	306	450
2428 ♀....	72	135	93	213	427
2445 ♀....	79	129	96	176	249
2461 ♀....	143	185	92	173	224
2463 ♀....	191	233	98	213	420

* It should be remembered in considering these data that the usual adult weight reached by our rats is: for females, about 200 gm.; males, about 300 gm. Many of the experiments were undertaken for different purposes than those here indicated and were stopped before the maximum possible weight was attained.

† These animals were allowed to grow very slowly but continuously on a diet low in protein.

‡ These animals died of lung disease which may have impaired their ability to attain a larger size.

The curve for Rat 2180 ♂ in the appended chart affords a striking example of the retention of the capacity to reach full adult size after a prolonged suppression of growth to the age of 303 days with food of low protein content. Resumption of growth was prompt and complete when the protein was increased to 18

per cent. The animal reached the unusually high maximum body weight of 376 gm. Partial statistics of two other rats (2104 ♀, 2112 ♀) which also reached full size after a similar earlier stunting during part of the time on 2 per cent lactalbumin food are given in the summary. Somewhat similar results with even lower protein concentrations in the food of early life were obtained with Rats 2342 ♂, 2321 ♀, and 2343 ♂ as shown in the summary. Even rats which had been fed, first for some time on a diet which furnished no nitrogen other than is contained in our "protein-free milk," and then on foods low in protein, were not damaged so as to prevent a subsequent renewal of growth to a size quite adequate for their sex (see page 451, Rats 2428 ♀ and 2445 ♀). The record of Rat 2463 ♀ illustrates the tenacity with which the capacity to grow is maintained unimpaired despite very severe earlier malnutrition. This animal received a diet containing as its protein addendum 2 per cent of gliadin, on which it lost 35 gm. during a period of 49 days. It was then maintained without further loss for 21 days on 2 per cent lactalbumin food. The exhibition of gradual increments in the content of this food protein, in successive stages of 4.5, 5, 7, and 9 per cent, resulted in satisfactory completion of growth to a size of 213 gm. Here we see resumption of growth on food which at best is not rich in protein, though the latter is of a superior quality as judged by our studies on the minimum protein requirement.[11]

A similar resumption of growth with 9 per cent edestin food is exhibited in the case of Rat 2113 ♀. The summary also contains indications of experiments in which the suppression was accomplished with diets low in casein (Rats 2116 ♀, 2051 ♂), and low in glycinin (Rat 2461 ♀) as the added protein.

In a number of instances we have conducted the feeding experiments so as to bring about a slow, prolonged growth, by furnishing a ration in which the relatively low content of protein has permitted a growth less rapid than that which is secured with a higher nitrogenous intake. *The period of slow growth may be greatly prolonged* in this way; *nevertheless appropriate adult size can ultimately be reached,* thus attesting the retention of an effective growth impulse and an absence of any impairment which

[11] Osborne and Mendel, *Jour. Biol. Chem.*, 1915, xxii, 241.

prevents the completion of the cycle leading to full size or body weight.

It should be noted that the resumption of growth has not been as perfect in every instance as in the typical records here presented. A positive result in these cases is far more valuable than a failure, because the latter may arise from a variety of extraneous, as well as inherent, causes which we cannot control or discover. In prolonged stunting, the animals may sometimes reach a precarious condition in which their vitality may become impaired beyond the possibility of recovery. They are sensitive to nocuous influences and cannot be expected to show great resistance under the conditions of limited diet. Subsequent statistics may show damage hitherto unappreciated. The factor of safety must be small.

SUMMARY AND CONCLUSIONS.

The growth impulse, or capacity to grow, can be retained and exercised at periods far beyond the age at which growth ordinarily ceases. In the case of our experimental animals, albino rats, in which increment of body weight ordinarily ceases before the age of 300 days, resumption and completion of growth were readily obtained at an age of more than 550 days. It is now reasonable to ask whether the capacity to grow can ever be lost unless it is exercised.

Even after *very prolonged* periods of suppression of growth, the rats can subsequently reach the *full size* characteristic of their species. In this respect there is no impairment of the individual.

The satisfactory resumption of growth can be attained not only after stunting by underfeeding, but also after the cessation of growth which results when the diet contains proteins unsuitable for the synthetic processes of growth or is low in protein.

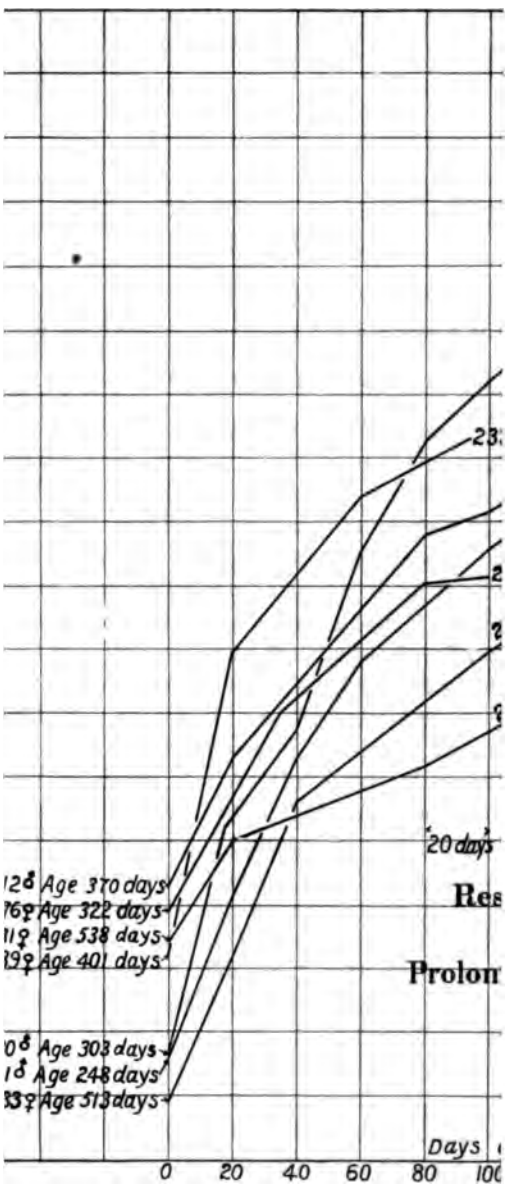
Growth in the cases referred to is resumed at a rate normal for the size of the animal at the time. It need not be slow, and frequently it actually exceeds the usual progress.

The size or age at which the inhibition of growth is effected does not alter the capacity to resume growth. Even when the suppression of growth is attempted for very long periods at a very small size (body weight) the restoration may be adequate when a suitable diet is furnished.

The procreative functions are not necessarily lost by prolonged failure to grow before the stage of development at which breeding is ordinarily possible.

The period of growth may be greatly prolonged by inadequacies in the diet, so that growth becomes very slow without being completely inhibited. Though the time of reaching full size is thus greatly delayed, growth, as expressed by suitable body weight can ultimately be completed even during the course of long continued retardation.

The methods of partially retarding or completely suppressing growth are too varied and unlike to permit final answers as yet regarding the outcome of all of the procedures of inhibition for the subsequent welfare of the individual. Our observations apply to the effects upon size and a few other incidental features mentioned. Although it is doubtful whether the fundamental features will be altered, far reaching dogmatic statements are scarcely justifiable until the experiments have been extended to include other factors and animal species.



ɜ. 5. Chart showing the increments of body we prolonged failure to grow. The curves of body ɔt indicated. As a rule they showed variations ls regarding the individual animals are given in t essed in these animals by a variety of procedu animals ultimately reached a body weight re size. The abscissæ represent days and the or

THE DETERMINATION OF UREA IN URINE BY THE UREASE METHOD.

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Marshall's[1] urease method, in the modification proposed by Van Slyke and Cullen,[2] is apparently the most accurate method available for the determination of urea. The method has, however, not yet attained the maximal degree of accuracy to be expected from volumetric processes in general. From the published figures of Van Slyke and Cullen, their modification appears to yield duplicates agreeing within about 1 per cent. Provided the removal of ammonia from the digestion mixture is assumed to be complete, the error must, in all probability, depend upon one of two factors. Either (1) the enzymotic reaction is not absolutely quantitative, or (2) traces of ammonia escape through the acid used for receiving it. As the result of an investigation of this point, the writer has found that, of these two possible sources of error, only the latter is present to a detectable extent. The aim of this paper is to present a further modification of the method which eliminates the above mentioned disturbance.

The greater degree of accuracy attainable by the modification to be described, as compared with those previously suggested, depends mainly upon four points.

1. By increasing the volume of fluid from which the ammonia is to be removed, and at the same time decreasing the concentration of potassium carbonate, it is possible so to regulate the rate of removal of the ammonia that every trace of it is held by the acid in the receiver (at the expense, of course, of a certain amount of time). When this part of the process is conducted as described below, it is possible to collect 7 mg. of ammonia nitrogen quanti-

[1] Marshall, E. K., Jr., *Jour. Biol. Chem.*, 1913, xiv, 283; 1913, xv, 495.

[2] Van Slyke, D. D., and Cullen, G. E., *ibid.*, 1914, xix, 211.

tatively in an amount of $\frac{N}{50}$ HCl which is hardly more than sufficient to neutralize it.

2. The aeration tube, after once being closed, is not again opened before the completion of the determination. Any chance of loss of ammonia is thereby eliminated.

3. The back titration is made with $\frac{N}{100}$ NaOH instead of $\frac{N}{50}$, using as the indicator methyl red,[3] which is sensitive to 0.05 cc. $\frac{N}{100}$ NaOH.

4. The minimum amount of urea recommended for the determination is the equivalent of approximately 25 cc. $\frac{N}{100}$ acid, the maximum about twice that amount. The error due to the titration itself is therefore only 0.1 to 0.2 per cent.

As the result of the above changes in technique, the figures obtained with pure urea solutions, by this method, agree with those obtained by the Kjeldahl method within 0.1 to 0.2 per cent. Duplicates on urine also agree within 0.1 to 0.2 per cent.

The Enzyme Solution.

Any satisfactory urease preparation can, of course, be used, provided it is standardized.[2] In this work aqueous soy bean extracts, prepared as described below, have been employed.

Extract 25 gm. of powdered soy beans for one hour with 250 cc. of distilled water, shaking at intervals. Add 25 cc. of $\frac{N}{10}$ HCl, and let stand 5 minutes. Filter with suction. To the filtrate add 5 cc. of a solution made by dissolving 70 gm. $Na_2HPO_4.12H_2O$ and 27 gm. KH_2PO_4 in 100 cc. of water. Keep in a cold place.

Blanks must be run on the extract at intervals of several days. In this laboratory, where a refrigerator kept at $-1°$ or $-2°$C. is available, extracts keep, without preservative, for as long as four weeks with but little loss of activity, and usually with no marked increase in ammonia content.

Method.

By means of an Ostwald pipette[4] transfer to a large test-tube (preferably heavy walled) an amount of urine containing from 3

[3] Methyl red was used for the micro-titration of ammonia by Pregl, F., Abderhalden's *Handb. d. biochem. Arbeitsmethoden*, 1912, v, 1346.

[4] Folin, O., and Farmer, C. J., *Jour. Biol. Chem.*, 1912, xi, 494.

to 6.5 mg. of urea nitrogen. Dilute with distilled water to a out 3 cc. Add 2 drops of kerosene and 2 cc. of the urease extract. Insert the rubber stopper bearing the aeration apparatus, and let stand for 15 minutes. Now add, by means of a pipette, the tip of which can be inserted into the air-inlet tube, 5 cc. of a carbonate-oxalate solution.[5] Aerate slowly for 5 minutes, then rapidly for 1 hour, collecting the ammonia in 25 cc. $\frac{N}{50}$ HCl contained in a narrow necked bottle of about 120 cc. capacity. Titrate the excess of acid with $\frac{N}{100}$ NaOH, using 2 or 3 drops of a 0.05 per cent alcoholic solution of methyl red. The end-point is the disappearance of the pink color.

The $\frac{N}{50}$ HCl and $\frac{N}{100}$ NaOH solutions used for the urea determinations recorded in this paper were carefully standardized against $\frac{N}{10}$ acid and alkali. Of the $\frac{N}{10}$ solutions, the HCl was standardized by the AgCl method, the NaOH by means of pure oxalic acid. The two $\frac{N}{10}$ solutions were checked against each other with excellent agreement, and were used in the Kjeldahl determination reported below. Calibrated glassware was used throughout.

Urea Solution.

A solution of Kahlbaum's urea was analyzed by the Kjeldahl method, and found to contain 1.743 mg. of nitrogen per cc. The following results were obtained with this solution by the urease method described above.

Urea solution.	$\frac{N}{100}$ HCl neutralized.	Urea N.	Urea N per cc.
cc.	*cc.*	*mg.*	*mg.*
2.0	24.86	3.483	1.741
	24.86	3.483	1.741
3.0	37.31	5.227	1.742
	37.25	5.219	1.740
4.0	49.68	6.960	1.740
	49.71	6.964	1.741

[5] This solution is prepared as follows: Dissolve 500 gm. K_2CO_3 in 500 cc. of water, with the aid of a little heat. Add 10 cc. of a 30 per cent solution of potassium oxalate. Filter if necessary, and let cool before using.

Urine.

Below are given the results of duplicate determinations obtained from *consecutive* samples of urine in the course of routine work.

Urine No.	Urea + ammonia nitrogen per 24 hrs.	Urine No.	Urea + ammonia nitrogen per 24 hrs.
	gm.		*gm.*
1	1.364	5	1.150
	1.362		1.149
2	1.325	6	1.151
	1.326		1.149
3	1.360	7	1.079
	1.361		1.081
4	2.460		
	2.458		

For many purposes, it is true, the additional accuracy obtain
by the above method, at the cost of a certain amount of ti
(but not of attention), is unnecessary. It must be admitte
however, that in some instances the greatest possible accuracy
essential, and in such cases the comparatively slight extra ti
required is negligible.

THE PREPARATION OF PROTEIN-FREE MILK.

By H. H. MITCHELL and R A. NELSON.

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(Received for publication, September 28, 1915.)

n nutrition investigations with white mice, now in progress, on substitution of protein by definite mixtures of isolated amino-ds, it was necessary to use rations containing only materials e from protein or protein derivatives, or containing these rogenous substances to a negligible degree. The carbohydrates l fats of the rations were easily amenable to this requirement, ce starch, dextrin, lactose, sucrose, lard, and purified butter could be obtained or prepared either nitrogen-free or with a nimal nitrogen content.

More difficulty was experienced with the mineral constituents ential for successful maintenance experiments of indefinite gth. None of the synthetic salt mixtures used by Osborne l Mendel[1] in their extensive experiments with albino rats has n very successful, apparently, in completely covering the neral requirements of these animals for long periods of time. hmann[2] obtained remarkably successful results with mice with artificial salt mixture, but the same mixture in experiments on s performed by Osborne and Mendel and in experiments on e undertaken by Wheeler,[3] has not given encouraging results. e discrepancy may rest in the fact that Röhmann did not use efully purified food substances, and, in particular, was content use commercial protein preparations.

McCollum and Davis have obtained very successful results h rats using rations containing an artificial salt mixture, the

Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington, blication No. 156*, pt. i, 1911.

Röhmann, F., *Biochem. Ztschr.*, 1914, lxiv, 30.

Wheeler, R., *Jour. Exper. Zool.*, 1913, xv, 209.

composition of which has recently[4] been published. Our experiences with this salt mixture in experiments on mice have not been particularly encouraging. Rations containing all their mineral constituents in this artificial preparation have invariably produced unmistakable symptoms of malnutrition in the experimental animals, generally after 2 or 3 weeks' time, though occasionally these symptoms have been deferred for a month, or even 2 months. The cause of the discrepancy between the results of McCollum and Davis and of this laboratory is still the subject of investigation. It is possible that it rests simply on the fact that different species of animals have been used. Another possible explanation is that the discrepancy is due to the use by McCollum and Davis of wood shavings in their experimental cages and of paper excelsior by ourselves. It is possible that in the former case the wood shavings may have furnished some indispensable mineral constituents absent from the artificial salt mixture which constituted a part of the rations.[5] This possibility is at present receiving attention.

In view of the lack of artificial salt mixtures of clearly established nutritive adequacy and of our own unsatisfactory results with perhaps the most promising salt mixture hitherto used in experimental work with small animals, our attention was naturally directed to the so called "protein-free milk" used with such success by Osborne and Mendel in covering the mineral requirements of rats for both maintenance and growth. The superiority of this "natural protein-free milk" over "artificial protein-free milk" preparations, containing lactose and salts in proportions

[4] McCollum, E. V., and Davis, M., *Jour. Biol. Chem.*, 1914, xix, 250.

[5] We cannot agree with the statement of McCollum and Davis that they "do not look upon the consumption of a small amount of wood fiber as objectionable to any greater degree in this type of experiment than is the feeding of agar-agar." Also, the availability and nutritive value of the nitrogenous and mineral substances of the wood cannot be as lightly disregarded, we believe, as they have been by these investigators.

The yellow pine shavings used in this laboratory in the comparative experiments with the McCollum and Davis salt mixture were found to contain 1.5 per cent of ash. The paper excelsior used in all of our work on the feeding of mice analyzed 0.80 per cent ash and 0.05 to 0.06 per cent nitrogen.

patterned as closely as possible after the chemical composition of the former preparation, has been shown by many experiments.[6]

Our experience with the Osborne and Mendel "protein-free milk" has been attended with marked success. For the maintenance of mice this preparation apparently satisfactorily covers all the mineral requirements, our work thus confirming that of Wheeler. The experiments of this character thus far undertaken in this laboratory have been almost entirely concerned with the nutritive requirements for maintenance. What little work we have done with growth requirements has indicated, also in agreement with the work of Wheeler, that with mice such requirements are different than with rats, though this difference is probably only of a quantitative nature.

From the nature of the investigations at present under way in this laboratory, some objection is involved in the use of "protein-free milk" as prepared by Osborne and Mendel, due to the fact that it is not entirely protein-free. The nitrogen content of this preparation averages about 0.7 per cent of the dry substance, of which about one-half is protein nitrogen.[7]

In our first attempt to reduce or eliminate the protein nitrogen in "protein-free milk" only a slight modification was made in the method of preparation. The original method involves precipitation of the casein with a slight excess of hydrochloric acid, filtration, heating the filtrate to boiling for one-half minute, filtering off the precipitated lactalbumin, neutralizing the clear filtrate, and evaporating to dryness at a temperature not exceeding 70°C. Upon neutralization of the filtrate obtained after separation of the lactalbumin, a precipitate is always obtained, probably consisting largely of calcium phosphate. In the hope that perhaps this precipitate would also contain residues of casein and possibly lactalbumin that had thus far escaped precipitation, it was filtered off, and the filtrate was evaporated to dryness as usual. In this way we have obtained preparations containing on an average about 0.10 per cent less nitrogen than the original Osborne and Mendel preparations. Presumably this reduction is largely, if not entirely, in the protein nitrogen. Thus, in one trial the casein and lactalbumin were precipitated from a sample of milk[8] and the

[6] Osborne and Mendel, *Jour. Biol. Chem.*, 1913, xv, 311.

[7] Osborne and Mendel, *Ztschr. f. physiol. Chem.*, 1912, lxxx, 316.

[8] It may be explained in this connection that all of our "protein-free milk" preparations were made from dried centrifugalized milk. This was dissolved in about twelve times its weight of distilled water in preparing "protein-free milk" either by the Osborne and Mendel procedure or by

filtrate was divided into two portions. One was neutralized with sodium hydroxide and immediately evaporated to dryness. The other was neutralized and filtered, and the clear filtrate evaporated to dryness. The first product contained 0.753 per cent nitrogen; the second 0.653 per cent. In another similar trial, the unfiltered product contained 0.614 per cent nitrogen, and the filtered product 0.494 per cent. The amount of nitrogen removed is really greater than these figures indicate, since the precipitate filtered off according to the modified procedure contains considerable inorganic material.

We have tested the nutritive value of the filtered "protein-free milk" preparations and have found that rations containing 28 per cent of the filtered product are apparently as effective (for periods of 5 or 6 months, at least) in covering the nutritive requirements of white mice for simple maintenance as the rations containing the same amount of the unfiltered product. This nutritive equivalence of the two preparations is interesting in view of the fact that the ash content of the unfiltered product has been reduced by the filtration introduced in the preparation of the filtered product, probably largely at the expense of the calcium phosphate.[9]

We have not tested the value of the filtered "protein-free milk" in covering the requirements for the normal growth of mice, to any great extent. The following experiment, however, is suggestive.

Two young male mice weighing 15.9 and 11.5 gm. were placed upon a ration containing 18 per cent casein, 26 per cent starch, 24 per cent lactose, 28 per cent lard, and 4 per cent of McCollum's salt mixture.[10] The weights of the mice slowly increased to maxima of 17.7 and 18.8 gm., respectively, in the course of about 50 days. From this time the weights gradually de-

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the modified procedure explained above. The product used is put on the market by the Merrill-Soule Co. of Syracuse, New York. The milk powder used in this work, taken from a 50 pound can, analyzed 6.03 per cent nitrogen, 6.21 per cent ash, and 2 per cent moisture. It is to be noted that the ash content is low, the normal figure being about 8.21 per cent. This undoubtedly accounts for the low ash values of our preparations.

[9] An analysis of one preparation of "protein-free milk" according to the Osborne and Mendel procedure gave 8.36 per cent ash. A filtered preparation gave 5.49 per cent ash.

[10] This is the equivalent of 3.7 per cent of the mixture containing anhydrous $MgSO_4$. In our rations we used $MgSO_4.7H_2O$ in quantities containing the same relative amount of magnesium as prescribed by McCollum.

clined, and other symptoms of malnutrition appeared; *i.e.*, roughened coats and decreased food intake. On the 83rd day of the experiment the mice weighed, respectively, 14.2 and 18.2 gm., and appeared to be in a miserable condition, the former much more so than the latter. At this time they were put upon a ration containing 18 per cent casein, 28 per cent of the filtered "protein-free milk," 28 per cent lard, and 26 per cent starch. In the course of 8 days the mice had increased in weight to 24.0 and 20.5 gm., respectively, and appeared to be in perfect condition.

Another method of reducing the nitrogen content of "protein-free milk" was suggested by the work of Greenwald[11] on the removal of the proteins of blood for the estimation of non-protein nitrogen. The reagent used was trichloroacetic acid.

According to the method proposed by Greenwald, the dilution of blood with nine volumes of 2.5 per cent trichloroacetic acid completely precipitated the proteins. This procedure would be entirely impracticable for the preparation of any considerable quantites of protein-free milk, on account of the excessive amount of added water that must be removed subsequently by evaporation. We therefore decided to test out a reagent containing 50 per cent of trichloroacetic acid, the first object of the test being to determine the amount of this reagent required for the complete removal of the proteins of milk. The second object was to ascertain the length of time that the filtrate must be boiled for the complete removal of the excess precipitant. This removal is effected, apparently, not so much by simple volatilization (trichloroacetic acid boils at a temperature of 195°C.), as by decomposition into carbon dioxide and chloroform, which are easily expelled from a boiling solution. We previously discovered that upon boiling the clear filtrates resulting from the removal of the precipitated proteins, a precipitate ultimately resulted. In this test, therefore, this precipitate was filtered off in order to determine its ash content and, indirectly, its nitrogen content.

Fifteen portions of 10 gm. each of the centrifugalized milk powder were each dissolved in 150 cc. of distilled water. They were divided into five sets of three portions each. To each portion of the first set, 2 cc. of a 50 per cent solution of trichloroacetic acid were added, to the second 6 cc. of this solution, to the third 12 cc., to the fourth 20 cc., and to the fifth 50 cc. After standing for ½ hour each portion was filtered and the precipitate

[11] Greenwald, I., *Jour. Biol. Chem.*, 1915, xxi, 61.

washed thoroughly with distilled water. Filtration was rapid, and g a water-clear filtrate. In each of the five sets, one portion was boiled 5 minutes, one portion for 30 minutes, and the third portion for 1 h Any coagulum formed during the boiling was then filtered off, weighed the filter paper, and ashed. The filtrates resulting were made up to cc. 100 cc. samples were then taken for the determination of total substance and ash, and for Kjeldahl nitrogen determinations. The sults of this complete test are given in the following table.

Amount of reagent used.	Length of boiling.	Coagulum obtained on boiling.		Filtrate from coagulum separated on boili Made up to 500 cc. and 100 cc. portions taken for analysis.*			
		Approximate dry weight.	Ash.	Dry weight.	Ash.	Nitrogen.	Nitr
cc.	*min.*	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*per*
2	5	0.219	0.0610	1.27	0.1322	8.65	0
"	30	0.318	0.2753	1.00	0.0683	6.97	0
"	60	Lost					
6	5	0.380	0.0040	1.42**	0.1280	9.19	
"	30	0.325	0.1769	1.21	0.0735	6.33	0 0
"	60	0.503	0.2363	1.17	0.0847	6.21	0 0
12	5	Practically none	0.000	1.55**	0.1057	6.57	
"	30	0.106	0.0062	1.30	0.1227	6.08	0 0.
"	60	Lost					
20	5	Practically none	0.0054	1.79**	0.1281	6.90	
"	30	0.024	0.0037	1.30	0.1365	6.35	0 .49
"	60	0.076	0.0052	1.30	0.1344	5.77	0 .44
50	5	0.093	0.0034			6.52	
"	30		0.0036	1.95**	0.1475	5.71	
"	60	0.103	0.0037	2.05**	0.1500	5.54	

* The data apply to the 100 cc. portions, and are equivalent to 2 gm. of milk powder.

** All the trichloroacetic was not removed during boiling.

The results in the third column on the dry weight of the coagulum obtained on boiling unfortunately are only approximate, since the coagulum was filtered and weighed on filter papers that were not tared. The weights given were obtained after subtracting the average weight of four filter papers of similar size and make. The greatest variation in weight among these four filter papers was 0.06 gm.

The results in the fifth column on the solids in one-fifth of the filtrate from the coagulum separated on boiling (representing 2 gm. of milk powder) are complicated by the fact that with some of the tests all the trichloroacetic acid was not removed during the boiling and subsequent evaporation on the water bath. These cases are indicated in the table by double asterisks.

The following conclusions seem justified from a study of these data:

1. When 2 cc. of the 50 per cent trichloroacetic acid solution were used per 10 gm. of milk powder, the excess of precipitant was removed by boiling for less than 5 minutes. Longer boiling caused a precipitation of amounts of mineral matter to some extent proportional to the period of boiling. When 6 cc. of reagent were used, the excess was not entirely removed by boiling for 5 minutes. Boiling for 30 to 60 minutes occasioned a precipitation of increasing amounts of mineral matter. When larger quantities of reagent were used no appreciable amount of mineral matter separated out even after 60 minutes' boiling. With the portions treated with 12 and 20 cc. of reagent, the excess of trichloroacetic acid was apparently completely removed by boiling for 30 minutes. With the portions treated with 50 cc. of reagent, even 60 minutes' boiling was not sufficient to remove completely the excess of acid.

2. The coagulum which separated on boiling contained organic as well as inorganic matter. For those portions treated with 12 cc. or more of the reagent, the coagulum was inconsiderable, containing a fairly constant amount of ash.

3. With all five series of tests, the longer the boiling the smaller was the quantity of nitrogen contained in the final filtrate.

4. In general the greater the quantity of reagent added the smaller was the amount of nitrogen in the final filtrate. This decrease in nitrogen, however, is insignificant for quantities of reagent greater than 12 cc.

In view of the difficulty of completely removing the larger quantities of trichloroacetic acid in a reasonably short time, and also of the greater expense involved in the use of such quantities, it was decided that the best procedure was the use of 12 cc. of 50 per cent trichloroacetic acid, per 10 gm. of milk powder, the filtrate being boiled for 30 minutes to 1 hour and filtered again.

Two separate preparations made according to this method contained 0.401 per cent nitrogen (boiled 30 minutes), and 0.420 per cent nitrogen (boiled 1 hour). The difference between these two results probably has no significance. The percentages of ash in the above two products were, respectively, 7.5 and 5.0 per cent. It is evident that boiling for one hour before filtration removes considerable mineral material without certainly removing more nitrogen. We would, therefore, recommend boiling for 30 minutes, filtering, and again boiling for 30 minutes for the complete removal of the excess trichloroacetic acid. The product thus obtained is similar in appearance and taste to preparations made according to the original Osborne and Mendel method.

A series of protein tests were run on the product containing 0.420 per cent nitrogen with the following results: Picric acid gave no precipitate; a large excess of trichloroacetic acid gave no precipitate; phosphotungstic acid gave a rather heavy precipitate on standing; tannic acid gave only a very slight precipitate, hardly more than a murkiness; potassium ferrocyanide and acetic acid gave no precipitate; with the biuret, Hopkins-Cole, and Millon reagents, no distinctive colorations were produced. These tests, therefore, failed to indicate with any degree of certainty the presence of protein material.

In the feeding experiments with trichloroacetic acid preparations of protein-free milk that have thus far been run, the product seems to be as efficient, for maintenance at least, as the original Osborne and Mendel preparations, though admittedly the tests are not of sufficient length to establish this point finally.

Two adult mice have been kept for 46 days on rations containing our protein-free milk and two others for 52 days, with maintenance of body weight and normal health and appetite. One mouse that had been kept for 22 days on a ration containing the McCollum salt mixture (with iodine given in the drinking water once a week), during which time it decreased in weight from 19.3 to 15.0 gm., recovered its original weight and normal appearance and behavior when changed to a ration similar to the first, with the exception that our protein-free milk was substituted for lactose and the McCollum salt mixture.

In view of the data given above on the low nitrogen content of protein-free milk prepared with the use of trichloroacetic acid as a protein precipitant, on the negative character of the protein tests made upon such preparations, and on its nutritive value in

covering the mineral requirements of mice for maintenance, we believe that the product should commend itself as a favorable substitute for the Osborne and Mendel product in experiments on rats and mice involving rations whose protein content must necessarily be under strict control. As admitted above, we are not yet in a position to conclude finally that our product will be adequate for experiments of indefinite duration, or that it can certainly be used to advantage in growth experiments.

Whether or not the nitrogen contained in protein-free milk prepared by the trichloroacetic acid method includes protein cleavage products, we hope to determine in the near future. The known presence of nitrogenous extractives, such as urea, creatinine, and purines, in milk in quantities—indirectly estimated, it is true—such that the extractive nitrogen would equal from 0.3 to 0.5 per cent of protein-free milk, establishes some probability that little or none of the nitrogen in our product is of nutritive value as a substitute for protein nitrogen.[12]

[12] The following calculation is of interest in view of Munk's conclusion quoted by Osborne and Mendel, that $\frac{1}{6}$ of the total nitrogen in milk is extractive in nature. 100 gm. of milk powder treated according to our method gave 63.26 gm. of protein-free milk. The nitrogen content of the milk powder was 6.03 per cent, and of the protein-free milk 0.40 per cent. Thus, the nitrogen in our protein-free milk may be shown to be equivalent to about $\frac{1}{13}$ of the total nitrogen of milk.

In a direct analysis of milk powder made in this laboratory by Mr. C. L. Newlin it was found that about $\frac{1}{12}$ of the nitrogen in this product is not precipitated by Stutzer's reagent.

THE TIME REQUIRED FOR REDUCTION OF OXYHEMOGLOBIN IN VIVO.

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If the spectroscope is directed to the red glare between adjacent fingers placed before a bright light, the two bands of oxyhemoglobin can with difficulty be detected. If now the wrist be tightly bandaged, the two bands can be seen fading away and being replaced by the single band of reduced hemoglobin. We particularly wished to know the time needed for the living tissues to effect this reduction of the blood pigment *in vivo*. We find that the time varies from 18 to 26 seconds, 18 seconds being the average of six consecutive observations in one series, and 26 the average of another similar series. We wished to be able to compare the time necessary to effect this reduction *in vivo* with that required by the reductase of fresh tissue press juice to reduce blood *in vitro* at 40°C. The shortest time cat liver juice needed was 2.5 minutes, which was also the time needed by triturated cortex cerebri of the cat. Pigeon muscle juice reduced oxyhemoglobin in 2 minutes, while pigeon liver juice and fish liver juice reduced it instantly. Vierordt in 1876 found that 40 seconds was the shortest time for the reduction of blood in the human finger; Henocque gave the time as 55 to 65 seconds.

The white ear of an albino rabbit also yields the two-banded spectrum; on compressing the base of the ear, the one-banded spectrum gradually appears. The average time necessary for this is of the order of 40 seconds, as in the following series of observations: 37, 44, 37, and 42 seconds. One of us[1] in 1897 found that the blood in the rabbit's ear was reduced after about 30 seconds' compression.

[1] Harris, D. F., *Proc. Roy. Soc. Edin.*, 1897–98, xxii, 195.

The spectrum of the two-banded pigment returns within 3 seconds after removing the constriction to the finger, ear, etc. This, of course, is not due to any phenomenon of reoxidation of the capillary blood, but is merely the mechanical result of the fresh arterial blood flooding the capillary district.

THE NON-DESTRUCTIBILITY OF URIC ACID IN THE HUMAN ORGANISM.

PRELIMINARY COMMUNICATION.

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(Received for publication, October 31, 1915.)

Schittenhelm and Wiener[1] examined human tissues for uric acid in three cases. Either no uric acid or only very small amounts could be demonstrated, from which they deduce further support for their long maintained contention that the human organism can decompose uric acid. The observations of Wiechowski[1] and others that in man 80 to 90 per cent of the parenterally introduced uric acid reappears in the urine, they attribute in part to the existence of a concomitant disturbance of nuclear metabolism resulting in an increased formation of uric acid, and in part to the probability that a portion of the uric acid so introduced is carried directly to the kidneys and never passes through the liver—an important organ for the metabolism of uric acid. They admit that human organs *in vitro* do not decompose uric acid, but ascribe this fact to the possible peculiarity of the enzyme involved (readily inactivated or destroyed). They argue that if uric acid is not destroyed in the human organism, the tissues should contain high concentrations of this compound, particularly in those instances where there is retarded elimination. Their results may bear review in some detail.

Case I.—Male, 62 years of age. Anuria for 6 days due to thrombosis of both renal veins following operation. Tissues obtained 6 hours post mortem. Secured for analysis: lung, 1,385 gm.; heart, 250 gm.; spleen, 250 gm.; liver, 1,785 gm. In each case 200 gm. samples were examined for uric acid, with entirely negative results. The remainder of the organs were worked up together from which was isolated but 0.01 gm. of uric acid.

Case II.—Girl, 16 years of age. Pernicious anemia. No uric acid could be demonstrated.

Case III.—Suffered with typical attacks of gout for 25 years, had many tophi in the ears. The whole organ was used for analysis in each case with the following results: liver (1,550 gm.), no uric acid; spleen (290 gm.), 10

[1] Schittenhelm, A., and Wiener, K., *Ztschr. f. d. ges. exper. Med.*, 1914, iii, 397.

mg. uric acid (3.5 mg. per 100 gm.); kidney (270 gm.), no uric acid; lung (930 gm.), 15 mg. uric acid (1.6 mg. per 100 gm.); muscle (440 gm.), no uric acid; and intestine (420 gm.), no uric acid.

The failure of Schittenhelm and Wiener to demonstrate uric acid in the cases of anuria and of gout is surprising. Their results may in part be ascribed to the use of hot sodium hydroxide previous to the precipitation of the proteins in the extraction of the tissues, as the instability of uric acid in alkaline solutions is a well known property.[2] The data recorded in the following tables present marked contrasts to those of Schittenhelm and Wiener reviewed above.

TABLE I.

Concentration of Uric Acid in Human Tissues and Fluids per 100 Gm. of Material.

Case.	E.E.* Uremia.	T.D. Uremia.	S.H. Uremia.	M.F. Diabetes.	C.M.† Diabetes.	S.T. Amputation.	H.‡ Pneumonia.
	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Blood	15.4	14.3	17.0	0.7	0.7	0.7	
Pleural fluid	16.7	15.9					
Ascitic fluid	18.0						
Pericardial fluid		14.3	18.0				
Subcutaneous fluid	18.0						
Spinal fluid	2.8	2.0	4.7				
Skeletal muscle	8.0	3.9	5.8	0.7	2.6	2.0	
Heart muscle	10.0	7.3	8.8		1.2		
Liver	18.0	15.6	11.5		5.0		4.0
Spleen	12.6	14.3	9.1		1.2		Trace
Skin		13.0					

* The data on this case have already been reported in another connection (Myers, V. C., and Fine, M. S., *Jour. Biol. Chem.*, 1915, xx, 396).

† This case died on Jan. 30, 1914. The ground samples of heart muscle, spleen, and liver were preserved in alcohol and refrigerated till Apr. 2, 1915, when the examinations were made.

§ This case died on Feb. 3, 1914. The tissues were preserved in alcohol and refrigerated till Apr. 5, 1915, when the examinations were made.

In the present study the method for determining uric acid in the tissues was essentially that employed for the blood,[3] with

[2] Compare Mitchell, P. H., *Jour. Biol. Chem.*, 1907, iii, 145; and more recently Folin, O., and Denis, W., *ibid.*, 1912-13, xiii, 469.

[3] Fine, M. S., and Chace, A. F., *Jour. Biol. Chem.*, 1915, xxi, 37?

TABLE II.

Concentration of Uric Acid in Miscellaneous Human Tissues per 100 Gm. of Material.

Tissue.	Uric acid.
	mg
Pectoral muscle	2.5
Uterine "	2.0
" "	2.5
" "	1.2
Mixed tonsils	1.7
Thyroid	0

slight modifications which will be described in a later communication.

The data recorded in Table I were obtained from material secured at autopsy. The fluids were in all cases analyzed fresh. The tissues from E. E. were obtained about 4 hours post mortem late in the afternoon, and placed in the refrigerator until the next morning. In the case of T. D. the tissues were worked up within 4 hours after death; and in that of S. H. the tissue samples were suspended in hot water within 3 hours after death.

As noted in the footnotes to Table I, some of the tissues in the cases C. M. and H. 7 were preserved for 14 months in strong alcohol at 0°C. before being analyzed. The objection may be raised that even this preservation is not adequate to inhibit autolysis completely.[4] It may be pointed out, however, that when liver samples of S. H. were allowed to autolyze with toluene for 145 days at 37°C. there was no noteworthy change in the uric acid concentration.

The data in Table II were all obtained at operation, the tissues being analyzed within 4 hours after removal from the body.

Comment with regard to the relative distribution of uric acid in body fluids and tissues will be withheld for the present. It is desired at this time merely to point out that, contrary to the findings of Schittenhelm and Wiener, *uric acid can be demonstrated in considerable concentrations in human tissues;* and that the contention, to which Schittenhelm and his coworkers have, for a long time, persistently adhered, finds no support in tissue analysis.

[4] Compare Wells, H. G., and Caldwell, G. T., *Jour. Biol. Chem.*, 1914, xix, 57.

A HYDROGEN ELECTRODE VESSEL.[1]

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INTRODUCTION.

With the extension of the use of the hydrogen electrode, especially in biochemistry, have come numerous modifications of the classic type of electrode vessel. The form here described was developed to meet some special requirements in a study of the hydrogen ion concentrations of bacterial cultures, and, although it embodies few radical improvements, its convenience and success are offered as sufficient apology for adding its description to the many now found in the literature.

The classic method of operating, in which hydrogen is bubbled through the solution in which a platinized electrode is wholly or partially immersed, proved useless in the studies referred to. With some culture media, even when electrode and solution were previously and separately saturated with hydrogen as recommended by Desha and Acree,[2] constant potentials were not observed until the lapse of several hours, during which period great changes may occur in active cultures. A rapid as well as accurate method was therefore demanded.

A quick attainment of constant potential, even in blood, has been shown by Michaelis and Rona[3] to be obtained if the platinized electrode, previously saturated with hydrogen, is allowed merely to touch the surface of the solution. This is probably due, as suggested by Hasselbalch,[4] and again by Konikoff,[5] to a

[1] Published by permission of the Secretary of Agriculture.

[2] Desha, L. J., and Acree, S. F., *Am. Chem. Jour.*, 1911, xlvi, 638.

[3] Michaelis, L., and Rona, P., *Biochem. Ztschr.*, 1909, xviii, 317.

[4] Hasselbalch, K. A., *Biochem. Ztschr.*, 1913, xlix, 451.

[5] Konikoff, A. P., *Biochem. Ztschr.*, 1913, li, 200.

rather sharply localized equilibrium at the point of contact. Reductions and gas interchanges having taken place within the small volume at the point of contact, diffusion from the remaining body of the solution is hindered by the density of the surface layer with which the electrode only comes in contact.

In exploring new fluids it appeared to the writer precarious to rely upon such a device, which appears to take advantage of only a localized and hence a pseudo-equilibrium, and which makes no allowance for a possible difference between the solution and surface film in the activity of the hydrogen ions. Hasselbalch's principle seemed therefore to be more suitable.

Hasselbalch[6] found that a very rapid attainment of a constant potential can be obtained by shaking the electrode vessel. Under these conditions there should be not only a more rapid interchange of gas between the solution, the gaseous hydrogen, and the electrode, an interchange whose rapidity Dolezalek[7] and Bose[8] consider necessary, but the combined or molecular oxygen, or its equivalent, in the whole solution should be more rapidly brought into contact with the electrode and there reduced. Furthermore, by periodically exposing the electrode the hydrogen is required to penetrate only a thin film of liquid before it is absorbed by the platinum black. The electrode should therefore act more rapidly as a hydrogen carrier. For these reasons a true equilibrium embracing the whole solution should be rapidly obtained with the shaking electrode; and indeed a constant potential is soon reached.

In Hasselbalch's design there appeared to the writer to be certain objectionable features. Aside from the rather clumsy dome, which Pauli[9] and also Manabe and Matula have replaced with a stopper, Hasselbalch's design and the various modifications which have since appeared maintain the long axis vertical. This, if complete rotation is not resorted to, requires a very wide angle of rotation to gain a maximum exposure of liquid surface. Mor

[6] Hasselbalch, *Biochem. Ztschr.*, 1911, xxx, 317; 1913, xlix, 451; *Comptes rend. trav. de Lab. Carlsberg*, 1911, x, 69.

[7] Dolezalek, F., *Ztschr. f. Electrochem.*, 1898-99, v, 533.

[8] Bose, E., *Ztschr. f. physikal. Chem.*, 1900, xxxiv, 701.

[9] Pauli, W., cited by Manabe, K., and Matula, J., *Biochem. Ztschr* 1913, lii, 369.

objectionable is the persistence in most of the published electrode vessels of dead spaces, especially in the tubes adjacent to the body of the vessels. Acree and Myers[10] state that constant potentials cannot be obtained so long as the portion of the solution used to connect with the KCl is left unsaturated with hydrogen.

It is unnecessary to speculate upon the quantitative significance of these and other errors in design, since they are easily eliminated as the following design will show.

The Vessel and Its Operation.

By setting up the chain in an air bath there are avoided many of the complicating details of design necessary in an oil bath. Theoretically an air bath is to be preferred if it can be kept at a fair degree of constant temperature, since air, because of its low specific heat, exchanges heat slowly with the objects it bathes. The purpose of a constant temperature bath is to keep not itself, but the apparatus it contains, at constant temperature. An air bath may be permitted to fluctuate in temperature more widely than an oil or water bath with the certainty that, if the fluctuations are regular and periodic as well as moderate, the apparatus will lag and remain very constant. The air bath used is well insulated with two inches of cork besides its well constructed frame and compo-board lining. Active as well as smooth circulation of the air is accomplished by fanning with a Sirocco fan from one end of a central box containing the apparatus, and by using the space between this box and the bath walls as flues for the return of the air to the other end. An ordinary fan is worthless in an air bath if smooth circulation is demanded. The heating unit is a length of bare nichrome No. 30 wire which retains very little heat when the current is stopped. The regulator is a grid containing about 60 cc. of pure mercury with the regulator head described in a previous paper.[11] When the regulator was first used periods were observed in which the temperature remained constant for periods of 8 hours within ± 0.002° (tapped Beckmann thermometer reading). This was a suspicious constancy. It soon settled down to about ± 0.05, but the variations from moment to moment were found by an eight pair copper-constan-

[10] Myers, C. N., and Acree, S. F., *Am. Chem. Jour.*, 1913, 1, 396.

tine thermo element to consist of very regular fluctuations of only ± 0.003, about a very slowly drifting mean. The present regulator has been in continuous use for a year and a half without attention.

In the manipulation of the vessel the purpose is first to bring every portion of the solution into intimate contact with electrode and hydrogen atmosphere, and then to draw the solution into a wide tube in forming the liquid contact.

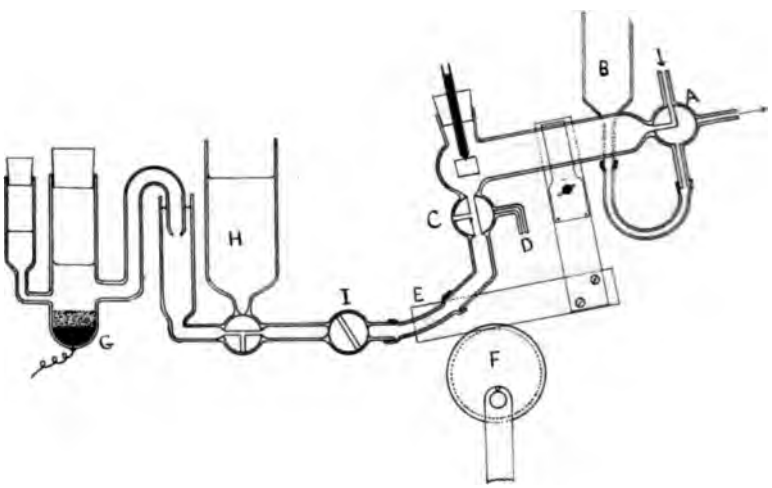

FIG. 1. Sectional view of the vessel and its accessory parts.

The electrode vessel is first flooded with hydrogen, of which there should be an abundant flow. Then, with the vessel rocked back until cock *A* is at its lowest point, the solution to be tested is run in from the small container *B*. It displaces hydrogen which wastes through *C* at *D*. Cock *C* is closed when the vessel is about ⅓ full of solution, and *A* is then turned as shown in the figure to allow a constant atmospheric pressure of hydrogen to bear upon the solution. The vessel is now rocked so that the electrode is alternately exposed to hydrogen and immersed in the solution.

[11] Clark, W. M., *Jour. Am. Chem. Soc.*, 1913, xxxv. 1889.

The rocking of the vessel is accomplished by supporting it upon a bar which is pivoted behind the flexible tube *E* and which rests in a groove of the eccentric wheel *F*. By changing the position of this wheel relative to the pivot of the bar the vessel can be given excursions of various ranges. The range should be wide enough to immerse and expose the electrode completely, and the speed of rocking should be such that the liquid actively but smoothly surges from end to end.

After the vessel has been rocked a sufficient length of time the solution must be brought into contact with the potassium chloride solution which completes the chain between the electrode and the calomel cell *G*. Before making this contact fresh KCl solution is allowed to flow from the reservoir *H* and waste at *D*. The flow is stopped by closing cock *I*. The flexible rubber tube *E* is then pinched; and, after turning *C* to connect with the vessel, the pinch is released. This brings the liquid contact below the constriction of the cock and makes it occur in a wide tube in accord with the recommendation of Cumming and Gilchrist.[12]

In the diagram the vessel and cocks are shown in the positions they occupy during a potential measurement. It will be noticed that the only cock in the chain which is closed is *I*. It is necessary to keep one cock closed to prevent the various solutions from being displaced from their proper positions. Cock *I* is chosen because it occurs in the best conducting liquid and does not separate dissimilar solutions. It should be left ungreased in the center; but, to prevent the creeping of KCl, and to insure ease in turning, the key is touched with vaselin at its widest part and the socket at its narrowest part. Then when the key is replaced it will ride in two rings of grease and with the central portion uncontaminated and filled with a good conducting film of potassium chloride solution.

In the specifications of this vessel it was demanded that the glass blower seal the cocks *A* and *C* to the body of the vessel as closely as possible and without constrictions at the junctures. When the stopper holding the electrode is forced down further than shown in Figure 1, the interior is left with practically no dead spaces for liquid or gas. By the use of the cocks the liquid within

[12] Cumming, A. C., and Gilchrist, E., *Tr. Faraday Soc.*, 1913, ix, 174.

the vessel is sharply separated from that without and no interchange can occur.

With the long axis horizontal the vessel needs only to be rocked slightly instead of rotated through a wide angle in order to expose a maximum solution surface and alternately expose and immerse the electrode. The gentleness of the required rocking not only prevents but tends to destroy the froth which is so easily produced in protein solutions.

With two such vessels mounted in parallel and connected through a three-way cock at *I* with the rest of the chain, determinations may be made with one while the other is being prepared for the next determination. This mounting also permits the measurement of one hydrogen electrode against another.

A word might be said here in regard to the operation of cock *C*. A study of the various positions of the key will show that if the portion of the bore which corresponds to the stem of a *T* is not filled before the liquid contact is formed it will remain unfilled and may give up a bubble of hydrogen which will be difficult to displace. This may be avoided by the following procedure: In filling the vessel the key of cock *C* is kept in the position ⊥. Before liquid contact is to be made *I* is opened and *C* is turned clockwise rapidly. With a little practice all the bores of the key are filled in this way without allowing any KCl to flow into the vessel. Mr. G. E. Cullen has suggested a three-way cock at C with outlets set at 120°. This permits the use of a two-way key with obvious advantage.

With the exception of a few cases such as measurements of acetate-acetic acid mixtures there has been no occasion to object to the use of a rubber stopper to hold the electrode. A glass stopper has not been used, partly because it would limit the choice of electrodes, partly to avoid the use of grease,[13] and partly because of the difficulty of grinding a stopper so that no capillary space will be left between its end and its socket to trap liquid. The rapidity with which measurements are taken reduces the influence of solution of the stopper or indeed of the glass. If the rubber must be protected a light coat of low melting point paraffin, warmed when the stopper is forced into place, will do.

[13] The surplus grease in each of the cocks was removed by a vigorous stream of solvent followed by alcohol and abundant water.

An adequate discussion of the accuracies attained with this vessel would involve a lengthy argument upon the values and reproducibilities of the various sources of potential in the whole chain. In the work so far reported[14] there has been no occasion to enter into this argument, since, by every criterion applied by others, the potentials were judged to be accurate within a millivolt, and no deduction based upon smaller differences has been presented. In this discussion we shall neglect corrections for barometric pressure, possible deviations of calomel electrodes, and estimates of the values of contact potentials. Only the observed potentials will be given to show the precision attained at the hydrogen electrode. The periods of measurement were too short and the hydrostatic pressure in the hydrogen generator too closely watched for variations in hydrogen pressure to affect the argument. Only one calomel cell was used in any one experiment and its deviation from other assumed standards was too small to consider. Potential measurements were made with a Leeds and Northrup type K potentiometer and the galvanometer customarily supplied with this potentiometer. The potentiometer was carefully calibrated by the Bureau of Standards. Two Bureau of Standards Weston cells furnished the known potential. The working standard was frequently compared with these, and the battery current was adjusted against this working standard at each potential measurement. The measured and measuring systems were electrically shielded against stray currents.

It is to be noted in the first place that if liquid contact is made at once so that the potential of the chain may be measured while equilibrium is being attained within the vessel, the potential of the chain rises rapidly. This may be seen in Table I. That this rise is due to processes at the electrode is evident from the fact that the maximum potential is almost immediately observed if the vessel is shaken for a short period before liquid contact is formed (Table II). In passing it may be said that the 1 per cent peptone solution used in these experiments has such a small buffer effect that the maximum observed variation in potential corresponds to that which would be produced by the addition of 0.0002 cc. normal acid or alkali to 10 cc.

[14] Clark, W. M., *Jour. Med. Research*, 1915, xxxi, 431; *Jour. Infect. Dis.*, 1915, xvii, 109. Clark, W. M., and Lubs, H. A., *Jour. Infect. Dis.*, 1915, xvii, 160. Clark, *Jour. Biol. Chem.*, 1915, xxii, 87.

In Table I a slight decline in potential is seen to have occurred after the maximum had been obtained. In this case the drift is thought to be located at the liquid contact and to be hastened by the disturbance due to shaking the vessel. In other cases the drift of the total potential of the chain has been toward a higher value. In every case this drift has been so slow, unless the liquid contact has been purposely and vigorously disturbed, its direction has varied so with different solutions, and its magnitude has been so small, that a clear tracing of its origin is difficult. It is believed to be located chiefly at the liquid contact. Therefore, in common with Lewis and Rupert[15] it is believed that better potentials are obtained directly after the formation of liquid contact.

Some experimenters continue observations over a long period and choose those potentials which remain constant during an arbitrarily selected central period. If under these circumstances there are drifts at electrode and liquid contact of opposite sign, the selection of a central period of constancy may be merely the selection of a period in which the opposite drifts have become approximately equal.

Since it is possible to reach equilibrium very rapidly at the electrode by the shaking method, and since drift at the liquid junction has been frequently reported by others and in some cases definitely traced while using the apparatus here described, it seems more reasonable to depend upon measurements continued for only a short time after the preliminary shaking, and to rely upon the reproducibility of several such measurements rather than upon the record of a single long experiment.

In the accompanying tables are given a few examples of measurements made with this vessel. Particularly interesting are the measurements of a culture medium containing 10 per cent gelatin. This was a stiff gel at lower temperatures, but at 30.0° it was sufficiently fluid to use in the electrode vessel. With no frothing of the solution satisfactory potentials were obtained within a remarkably short time. Equally satisfactory results were obtained with high fat milks.

So far there has been no occasion to investigate solutions of

[15] Lewis, G. N., and Rupert, F. F., *Jour. Am. Chem. Soc.*, 1911, xxxiii, 299.

low hydrogen ion concentration containing CO_2. For such solutions the vessel should do quite as well as Hasselbalch's, since its principles are the same.

In conclusion it must be admitted that no comparisons other than the one alluded to in the introduction have been made between the vessel here described and other designs. Therefore no proof of superiority can be advanced for the new design. Occasionally in the course of several thousand potential measurements the results have not been as satisfactory as those reported in the tables. Occasionally results have shown better agreement. Those reported are fairly representative of the kind of results which careful manipulation will bring; and, when the time within which they were obtained is taken into consideration, it may be said that they are satisfactory for all ordinary purposes. Before closer agreement can have any significance a great deal of more fundamental work will have to be done upon several problems such as that of liquid contact potential.

TABLE I.

Showing rise in total potential during shaking.

Chain: Hg HgCl | Saturated KCl | 1 per cent peptone solution | Pt H_2

Liquid contact formed from the first. Shaking continued between measurements.

Vessel.	Time.	Potential.
	min.	*volts*
A	0	0.6490
	1	0.6680
	2	0.6729
	4	0.6740
	5	0.6740
	9	0.6737
	11	0.6736
	13	0.6737
	17	0.6735
B	0	0.6325
	1.5	0.6653
	4	0.6735
	6	0.6737
	8	0.6736
	10	0.6734

TABLE II.

Potential measurements of a 1 per cent peptone solution. Prelimina [redacted] shaking 5 minutes. Vessel not shaken after the formation of liquid co [redacted] tact.

Chain: Hg HgCl | Saturated KCl | 1 per cent peptone solution | Pt H[redacted]

Vessel.	Time.	Potential.
	min.	*volts*
A	1	0.6736
	2	0.6738
	4	0.6738
	5	0.6738
B	1	0.6742
	2	0.6742
	4	0.6742
	5	0.6742
A	1	0.6737
	2	0.6739
	4	0.6739
	5	0.6739
B	1	0.6736
	2	0.6738
	4	0.6737
	5	0.6738
Average		0.6739

TABLE III.

Potentials of the chain:

Hg HgCl | Saturated KCl | Borate-boric acid mixture | Pt H_2

No shaking between measurements.

Vessel.	Preliminary shaking.	Time of measurement.	Potential.
	min.	*min.*	*volts*
A	5	1	0.80039
		5	0.80040
		12	0.80040
B	7	1	(0.80025)
		5	(0.80025)
A	10	1	0.80030
		20	0.80033
B	10	1	0.80034
		15	0.80034
Average			0.80036

TABLE IV.

Potentials of the chain:

Hg HgCl | Saturated KCl | Phosphate mixture | Pt H_2

No shaking between measurements.

Vessel.	Preliminary shaking.	Time of measurement.	Potential.
	min.	*min.*	*volts*
A.....	11	1	0.64235
		7	0.64240
		11	0.64238
B.....	9	1	0.64239
		3	0.64245
		12	0.64240
A.....	9	1	0.64249
		13	0.64245
B.....	13	1	0.64249
		9	0.64250
Average.....			0.64243

TABLE V

Potentials of the chain:

Hg HgCl | $\frac{N}{10}$ KCl | Saturated KCl | Gelatin culture medium | Pt H_2

Preliminary shaking 10 minutes. No shaking between measurements.

Vessel.	Time.	Potential.
	min.	*volts*
A.....	5	0.7420
	10	0.7417
	15	0.7417
B.....	5	0.7411
	10	0.7419
	15	0.7419

TABLE VI.

Potentials of the chain:

Hg HgCl | $\frac{N}{10}$ KCl | Saturated KCl | Milk | Pt H_2

Preliminary shaking 5 minutes. No shaking between measurements.

Vessel.	Time.	Potential.
	min.	*volts*
A.....	5	0.7304
	10	0.7304
B.....	5	0.7303
	10	0.7303

TABLE VII.

Potentials of the chain:
Hg HgCl | $\frac{N}{10}$ KCl | Saturated KCl | Meat infusion medium | Pt H_2
Preliminary shaking 5 minutes. No shaking between measurements.

Vessel.	Time.	Potential.
	min.	*volts*
A	1	0.7014
	5	0.7014
B	1	0.7013
	3	0.7014
	13	0.7014
	28	0.7014
	35	0.7013

TABLE VIII.

Potentials of the chain:
Hg HgCl | $\frac{N}{10}$ KCl | Saturated KCl | 5 day culture of *B. coli* in 1 per cent peptone 1 per cent dextrose | Pt H_2
Preliminary shaking 5 minutes. No shaking between measurements.

Organism.	Time.	Potential.
	min.	*volts*
hw	5	0.5970
	10	0.5970
	15	0.5970
hx	5	0.5960
	10	0.5960
hy	5	0.5937
	10	0.5958
	15	0.5958
hz	5	0.5947
	10	0.5956
	15	0.5956
	20	0.5956
fg	5	0.5942
	10	0.5960
	15	0.5962
	20	0.5962

THE REFRACTIVE INDICES OF SOLUTIONS OF CERTAIN PROTEINS.

IX. EDESTIN.

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(Received for publication, October 27, 1915.)

It has been shown by Robertson[1] that if proteins are dissolved in alkaline or acid aqueous solutions the change in the refractive index of the solvent is directly proportional to the concentration of the dissolved protein. This relation can be expressed by the equation

$$n - n_1 = a \times c$$

where n is the observed refractive index of the protein solution, n_1 that of the solvent in which the protein is dissolved, c the percentage of the protein in solution, and a a constant, expressing the change in the refractive index of the solvent by the addition of 1 gm. of protein per 100 cc. The increase in the refractive index is independent of temperature between the limits studied; *viz.*, 20° and 40°C. The constant a has thus far been determined for casein,[2] ovomucoid and ovovitellin,[3] paranuclein,[4] serum globulin,[5] casein in alcohol-water mixtures,[6] gliadin,[7] ox serum,[8] salmine,[9] globin,[10] and a compound protein, globin caseinate.[11]

[1] Robertson, T. B., *Jour. Phys. Chem.*, 1909, xiii, 469; Die physikalische Chemie der Proteine, Dresden, 1912, 317.

[2] Robertson, *Jour. Phys. Chem.*, 1909, xiii, 473.

[3] Robertson, *Jour. Biol. Chem.*, 1909–10, vii, 359.

[4] Robertson, *ibid.*, 1910–11, viii, 287.

[5] Robertson, *ibid.*, 1910–11, viii, 441.

[6] Robertson, *ibid.*, 1910–11, viii, 507.

[7] Robertson, T. B., and Greaves, J. E., *ibid.*, 1911, ix, 181.

[8] Robertson, *ibid.*, 1912, xi, 179.

[9] Robertson, *ibid.*, 1912, xi, 307.

[10] Robertson, *ibid.*, 1912–13, xiii, 455.

[11] Robertson, *ibid.*, 1912–13, xiii, 499.

Use of the refractometer has also been made to determine proteins quantitatively,[12] such determinations depending upon the previous knowledge of the value of a for the particular protein to be estimated.

While preparing a considerable amount of edestin for other purposes than herein given, it was considered of interest to determine its refractive index in various solvents. Edestin is of particular interest inasmuch as it can be prepared in a very pure state, and also on account of the large amount of work done with this protein by various workers.

Edestin was prepared according to the method given by Osborne[13] and used by Chittenden and Mendel,[14] Leipziger,[15] and others. Edestin prepared in this manner consists, as shown by Osborne,[16] of a mixture of edestin mono- and dichloride and is acid towards phenolphthalein. A very small amount failed to dissolve in 10 per cent sodium chloride solution, an observation also made by Weyl,[17] Chittenden and Mendel,[18] and Starke,[19] and explained by Osborne[20] as resulting from the hydrolytic action of the hydrogen ions of water whereby edestan is formed. In the acid and alkaline solutions used, the preparation was completely soluble. On ignition, a negligible quantity of ash remained.

Since the change in the refractive index of a solvent, on adding any given amount of solute, depends on the *size* of the molecules

[12] Reiss, E., *Arch. f. exper. Path. u. Pharm.*, 1904, li, 18; *Beitr. z. chem. Phys. u. Path.*, 1904, iv, 150. Robertson, *Jour. Ind. and Engin. Chem.*, 1909, i, 723; *Jour. Phys. Chem.*, 1910, xiv, 377; *Jour. Biol. Chem.*, 1912, xi, 179; 1912, xii, 23; 1912–13, xiii, 325; 1913, xiv, 237. Woolsey, J. H., *ibid.*, 1913, xiv, 433. Wells, C. E., *ibid.*, 1913, xv, 37. Buck, L. W., *Jour. Pharm. and Exper. Therap.*, 1913–14, v, 553. Thompson, W. B., *Jour. Biol. Chem.*, 1915, xx, 1. Briggs, R. S., *ibid.*, 1915, xx, 7. Robertson, *ibid.*, 1915, xxi, 233. Tranter, C. L., and Rowe, A. H., *Jour. Am. Med. Assn.*, 191[illegible], lxv, 1433.

[13] Osborne, T. B., *Am. Chem. Jour.*, 1892, xiv, 671.

[14] Chittenden, R. H., and Mendel, L. B., *Jour. Physiol.*, 1894–95, xvi, 48.

[15] Leipziger, R., *Arch. f. d. ges. Physiol.*, 1899, lxxviii, 402.

[16] Osborne, *Jour. Am. Chem. Soc.*, 1902, xxiv, 39.

[17] Weyl, T., *Ztschr. f. physiol. Chem.*, 1877–78, i, 72.

[18] Chittenden and Mendel, *Jour. Physiol.*, 1894-95, xvii, 48.

[19] Starke, J., *Ztschr. f. Biol.*, 1900, xl, 419.

[20] Osborne, *Jour. Am. Chem. Soc.*, 1902, xxiv, 28.

of the solute, and since in the case of edestin the molecule is large (molecular weight given by Osborne[21] as 14,500) only a slight error, probably less than the experimental, is made by using a mixture of the chlorides of edestin instead of the free base. However, in preparing a solution of edestin in any given concentration of acid or alkali, a correction for the acid combined with the base was made, this being readily determined by titrating with alkali, using phenolphthalein as an indicator.

2 per cent solutions of edestin in various concentrations of acids and alkalies were made, filtered to insure perfectly clear solutions, and dilutions to 1.5, 1, and 0.5 per cent made. The refractive indices of these solutions were then made, at the stated temperatures, in a Pulfrich refractometer reading to within 1′ of the angle of total reflection. A sodium flame was employed as the source of light. All solutions were adjusted to the temperature of the room so that the temperature of the refractometer prism and that of the solutions used would be the same. Calculations of the refractive index were made from a table supplied by the maker of the instrument. It was found that by checking the instrument with distilled water and known solutions, the true refractive index as given by the table was not obtained, but differed by a constant (about 50′). The values for the refractive indices given in the table are not to be taken as absolute. For the purpose of this paper we are concerned merely with the difference between the refractive index of solution and solvent, hence absolute values are unnecessary.

In the following table are given the results obtained. The values for a are calculated from the equation $n - n_1 = a \times c$.

From the table it will be seen that within the limits of the experimental error and for the different solvents used, the value for a is a constant. Since the experimental error, reading to within 1′ on the refractometer, is greatest in the most dilute solutions and least in the most concentrated, it would be incorrect in obtaining the most probable value for a merely to average the values for a as calculated from each determination. As shown by Robertson[22] the correct method of obtaining the value for a

[21] Osborne, *Jour. Am. Chem. Soc.*, 1902, xxiv, 77.
[22] Robertson, *Jour. Biol. Chem.*, 1910–11, viii, 510.

TABLE I.

Concentration of edestin.	Refractive index.	a.	Possible error for a
	Solvent $\frac{N}{50}$ KOH. *Temperature 18.5°C.*		
per cent			
0	1.32948		
0.5	1.33039	0.00182	±0.00016
1.0	1.33124	0.00176	±0.00008
1.2	1.33162	0.00178	±0.00007
2.0	1.33309	0.00180	±0.00004
	Solvent $\frac{N}{20}$ KOH. *Temperature 18.5°C.*		
0	1.32986		
0.5	1.33078	0.00184	±0.00016
1.0	1.33162	0.00176	±0.00008
1.2	1.33201	0.00179	±0.00007
2.0	1.33340	0.00177	±0.00004
	Solvent $\frac{N}{10}$ KOH. *Temperature 16°C.*		
0	1.33070		
0.5	1.33155	0.00170	±0.00016
1.0	1.33247	0.00177	±0.00008
1.5	1.33332	0.00175	±0.00005
2.0	1.33418	0.00174	±0.00004
	Solvent $\frac{N}{10}$ NH_4OH. *Temperature 19.5°C.*		
0	1.32955		
0.5	1.33039	0.00168	±0.00016
1.0	1.33124	0.00169	±0.00008
1.5	1.33216	0.00174	±0.00005
2.0	1.33293	0.00169	±0.00004
	Solvent $\frac{N}{10}$ Na_2CO_3. *Temperature 20°C.*		
0	1.33024		
0.5	1.33108	0.00168	±0.0001
1.0	1.33193	0.00169	±0.0000
1.5	1.33278	0.00169	±0.0000
2.0	1.33356	0.00166	±0.000

TABLE I—*Concluded.*

ncentration of edestin.	Refractive index.	*a*.	Possible error for *a*.
	Solvent $\frac{N}{50}$ HCl. *Temperature 17°C.*		
per cent			
0	1.32963		
0.5	1.33054	0.00182	±0.00016
1.0	1.33139	0.00176	±0.00008
1.5	1.33232	0.00179	±0.00005
2.0	1.33309	0.00173	±0.00004
	Solvent $\frac{N}{20}$ HCl. *Temperature 18°C.*		
0	1.32971		
0.5	1.33054	0.00166	±0.00016
1.0	1.33139	0.00168	±0.00008
1.5	1.33224	0.00169	±0.00005
2.0	1.33309	0.00169	±0.00004
	Solvent $\frac{N}{10}$ HCl. *Temperature 19.5°C.*		
0	1.33016		
0.5	1.33101	0.00170	±0.00016
1.0	1.33185	0.00169	±0.00008
1.5	1.33262	0.00164	±0.00005
2.0	1.33340	0.00162	±0.00004
	Solvent $\frac{N}{10}$ $HC_2H_3O_2$. *Temperature 20.5°C.*		
0	1.32963		
0.5	1.33047	0.00168	±0.00016
1.0	1.33131	0.00168	±0.00008
1.5	1.33216	0.00169	±0.00005
2.0	1.33309	0.00173	±0.00004
	Solvent 10 per cent NaCl. *Temperature 26°C.*		
0	1.34436		
0.5	1.34519	0.00166	±0.00016

is to add all the values for $n - n_1$ and divide this total by the total of the concentrations employed. A similar procedure is used in calculating the possible error for the value a. Proceeding in this way we obtain from all the determinations made, the average value for a as 0.00174 ± 0.00006.

It is interesting to observe that in acid solutions of edestin the value of a does not differ in magnitude from the value found in alkaline solutions, despite the fact that in dilute acid solutions the greater part of the edestin is converted, as has been shown by Osborne,[23] into edestan, this latter substance being the first product of the hydrolytic cleavage of the globulin, edestin, and forming salts with hydrochloric acid corresponding to a trichloride. Robertson[24] has shown that the complete hydrolysis of sodium caseinate by trypsin does not alter the refractive index of the solutions; *i.e.*, the value for a remains constant. This means that the refractive index of a solution containing the split products of a protein is the algebraic sum of the components and equal to the refractivity of the whole, since the volume of the solution remains constant. This is also true in the case of a compound protein, globin caseinate, and of the mixed proteins of the blood sera, as has been shown by Robertson.[25]

In the case of edestin, as is also true of the other proteins studied, the increase in the refractive index of an aqueous solvent (acid, base, or salt) due to any given concentration of a dissolved protein is independent of the nature and concentration (within the limits studied) of the aqueous solvent. This is to be expected since refractivity is dependent on the molecular volume or the sum of its component atoms. Since the protein molecule is enormously large as compared with the hydrogen, chlorine, or potassium atom, it can readily be understood that the substitution of a potassium for a hydrogen atom, or *vice versa*, has no measurable influence on the refractivity of any given concentration of protein solution.[26]

[23] Osborne, *Jour. Am. Chem. Soc.*, 1902, xxiv, 28.

[24] Robertson, *Jour. Biol. Chem.*, 1912, xii, 23.

[25] Robertson, *Jour. Biol. Chem.*, 1912-13, xiii, 505; 1912, xi, 179.

[26] For a complete discussion of this subject, see Robertson, Die physikalische Chemie der Proteine, Dresden, 1912, 319.

SUMMARY.

1. The refractive indices of varying amounts of edestin, dissolved in various concentrations of solutions of acids, bases, and salts, have been measured.

2. The solutions of edestin were found to follow the law $n - n_1 = a \times c$, where n is the observed refractive index of the protein solution, n_1 that of the aqueous solvent, c the percentage of dissolved protein, and a a constant expressing the change in the refractive index of the aqueous solvent by the addition of 1 per cent of protein.

3. The average value for a in the above formula was found to be 0.00174 ± 0.00006.

4. The value for a remains constant even though the solvent causes hydrolysis of the dissolved protein.

I am indebted to Professor T. Brailsford Robertson for the constant interest that he has taken in this work, and to the George Williams Hooper Foundation for Medical Research for financial aid in carrying out the investigation.

NOTE ON THE REDUCTION OF OXYHAEMOCYANIN IN THE SERUM OF LIMULUS POLYPHEMUS L.

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It is a well known phenomenon that when the blue blood drawn from certain Mollusca, Crustacea, and Arachnida is allowed to remain undisturbed in a tall vessel, after a time, varying according to species and temperature, it begins to decolorize at the bottom of the containing vessel, the blue color fading progressively upward until only a narrow layer at the surface retains its color. This change in color is due to the reduction of the blue oxyhaemocyanin contained in the blood.

This phenomenon in the blood of *Limulus polyphemus* was first studied by Gotch and Laws.[1] They reported that, when experimenting with whole blood, no satisfactory blanching of the liquid was observed when a stream of carbonic acid gas was passed through it, but that serum was blanched at once by the addition of ammonium sulphide. Howell,[2] who made similar studies at about the same time, stated that passing a stream of carbonic acid gas through serum of *Limulus* does not decolorize it. Kobert[3] studied this phenomenon in the blood of the cephalopod, *Eledone moschata*, and found that when preserved in a sterile condition out of contact with air, this blood was not decolorized even at a temperature of 39-40°C., provided reducing substances, such as sugars, were absent. Phisalix[4] investigated the same phenomenon in the blood of the vineyard snail, *Helix pomatia*. In whole blood of this animal reduction was checked by the addition of chloroform, ether, formaldehyde, or sodium fluoride, by the careful addi-

[1] Gotch, F., and Laws, J. P., On the Blood of *Limulus polyphemus*, *British Assn. Adv. Sc., Report*, 1884, liv, 774.

[2] Howell, W. H., Observations on the Chemical Composition and Coagulation of the Blood of *Limulus polyphemus*, *Johns Hopkins Univ. Circular V*, 1885, 4.

[3] Kobert, R., Ueber Hämocyanin nebst einigen Notizen über Hämerythrin, *Arch. f. d. ges. Physiol.*, 1903, xcviii, 411.

[4] Phisalix C., Observations sur le sang de l'escargot (*Helix pomatia*) Réduction de l'hémocyanine, *Compt. rend. Soc. de biol.*, 1900, lii, 729.

tion of acetic acid, by removing the floccules resulting from incomplete saturation with magnesium sulphate or with sodium chloride, by chilling in chipped ice, by repeated heating to 65°C., and, finally, by dialysis even though the blood cells were not removed and bacteria grew. If the material in the dialysate was returned to the dialyzed blood, reduction did not take place. Destroying the cells by heating for 20 minutes at 60°C. and repeating after an interval of 24 hours did not prevent reduction. Filtering blood through a Chamberland filter gave a clear, colorless filtrate, while a thick blue liquid remained on the filter which decolorized more rapidly than did the original blood. Saturation with magnesium sulphate yielded a precipitate, which, when redissolved in water, formed a solution that gradually decolorized. If such a solution was dialyzed, the fading was diminished. Phisalix concluded that the reduction was due to the presence of protein substances and not to microorganisms. His conclusions were based on the fact that fading, or reduction, occurred in blood which had been heated to 60°C., and which could, therefore, contain no living cells. The blood cells must have been destroyed by the heat, and microorganisms were assumed to be absent since the blood remained clear. Moreover, dialysis prevented the fading or reduction. It was assumed that dialysis did not affect the cells or the microorganisms. Phisalix also found that adding to the serum ammonium oxalate in excess of that necessary to precipitate the calcium hastens fading. Further, he found that serum thus decolorized may not, when shaken with air, completely recover its color. Since Warburg[5] has shown that the blood cells in vertebrate blood exert reducing power, it may be safely believed that in whole blood of *Helix* the cells have more or less reducing power.

Further, Heffter[6] has shown that one class of reductions depends upon the cleavage of the thio group from the protein, the reductions being greatly accelerated by heat.

The present paper contributes some additional observations concerning the reduction of haemocyanin of the blood of *Limulus polyphemus* L. In making the observations recorded below, the whole blood of *Limulus polyphemus* could not be employed because it is possible only with difficulty to prevent its clotting. The serum of *Limulus* used was free from cells, being prepared as described by Alsberg and Clark.[7] Before examination at Woods

[5] Warburg, O., Über Beeinflussung der Oxydationen in lebenden Zellen nach Versuchen an roten Blutkörperchen, *Ztschr. f. physiol. Chem.*, 1910, lxix, 452.

[6] Heffter, A., Die reduzierenden Bestandteile der Zellen, *Med.-Naturwissensch. Arch.*, 1907, i, 81; Giebt es reduzierende Fermente im Tierkörper? *Arch. f. exper. Path. u. Pharmakol.*, 1908, Suppl., 253.

[7] Alsberg, C. L., and Clark, E. D., The Haemocyanin of *Limulus polyphemus*, *Jour. Biol. Chem.*, 1910, viii, 1.

Hole or shipment to the laboratory, some of the animals, from which the serum was drawn, were bled at once after collecting, others remained in a floating car in the harbor for 6 weeks in certain cases, and 10 weeks in other cases.

The following tests were made with definite portions of prepared serum of *Limulus* varying in amount from 5 to 10 cc.

When the serum was chilled to temperatures near 0°C., the spontaneous reduction of the haemocyanin was greatly checked. When heated, reduction was greatly augmented, the maximum effect being obtained at about 30°C. Warming the serum to

TABLE I.

The Reducing Action of the Serum of Limulus on Various Reagents.

No reduction.	Slight reduction.
Cerulein	Alizarin disodium sulphonate
Crystal violet	Indigo carmine
Gallocyanin	Phenolphthalein
Malachite green	Potassium ferricyanide
Mercurous chloride	
Methylene blue	
Methyl orange	
Nitrobenzene	
Potassium picrate	
Sodium nitrate	
" nitroprusside	
" selenite	
" telluride	
Sulphur	

40°C. for 5 or 10 minutes inhibited reduction almost completely. Warming to 60°C. inhibited absolutely with the formation of an insignificant coagulum. In Table I are grouped the various reagents showing no reduction and those showing slight reduction in the presence of the serum of *Limulus*.

Serum of *Limulus* does not reduce sodium nitroprusside, a reaction said by Heffter to be characteristic of labile thio groups. Flowers of sulphur left in contact with the serum for many hours do not evolve any traces of hydrogen sulphide when subsequently acidified. On heating after acidifying neither lead acetate paper nor polished silver foil are blackened in the vapors. *Limulus*

serum, therefore, does not behave like the "Philothion" of de Rey-Pailhade.[8]

While no evidence was obtained that the thio group is responsible for the reduction of oxyhaemocyanin in blood allowed to stand, hydrogen sulphide passed into the serum at room temperature decolorized it promptly, producing at the same time a slightly dirty, yellowish tint. Serum decolorized in this way regained its color promptly when shaken in air, but lost it again very quickly and continued to behave in this way for several days, probably because of the absorbed hydrogen sulphide. At the same time it acquired a faintly brownish tinge, as though a part of the copper were being converted into the sulphide.

When gases like hydrogen, illuminating gas, or carbonic acid gas, were passed through the blue serum, at first there was no change of color. After 30 or 40 minutes fading began but was never complete. The time that must elapse was so long that it seemed probable the passage of the gas merely hastened slightly, if at all, the ordinary spontaneous fading. Serum decolorized in this way turned blue when shaken in air, but did not again fade quickly.

The antiseptics, chloroform and toluene, inhibited spontaneous fading. To get the effect with toluene the serum was thoroughly shaken with it. The protective action of the layer of toluene, keeping the serum out of contact with the atmosphere, is not a factor, for a layer of neutral cottonseed oil several inches thick did not interfere with the decolorizing. The effect of toluene, like that of many other agents that prevent reduction, was not permanent. After standing undisturbed several days under toluene the serum may begin to fade.

The effect of dialysis on the serum is not easy to judge, because haemocyanin of *Limulus*, like globulins in general, is, in part, precipitated by dialysis.[7] The rate of reduction was greatly diminished. On the other hand, the loss of salts and other dialyzable matter also seemed to have some influence, for, when the normal alkalinity was restored to thoroughly dialyzed serum and sufficient pure sodium chloride added to produce solution,

[8] de Rey-Pailhade, J., Sur un corps d'origine organique hydrogénant le soufre à froid, *Compt. rend. Acad. d. sc.*, 1888, cvi, 1683.

the normal rate of reduction was not restored. Reduction will, however, occur in dialyzed serum after standing some days in the ice box or after 36 hours at room temperature.

Small amounts of iodine or bromine water did not affect the fading. The addition of a considerable quantity of ammonium sulphate inhibited the reduction. If, however, enough was added to produce precipitation the haemocyanin separated in the form of pale blue floccules of lighter blue color than the serum before the treatment with salt. Possibly, when precipitated in this fashion, the haemocyanin does not have its maximum oxygen content, for, when it is removed from the solution by filtration, the surface of the precipitate on the filter paper, where exposed to the air, becomes a blue of darker color than the material underneath. If the material was dissolved in a sufficiently small amount of water no fading occurred. After 12 to 48 hours, varying with the temperature, a certain amount of decolorizing occurred if the salt was sufficiently diluted with water.

The reducing power of the serum, if any, was found to be very slight. Sodium telluride, sodium selenite, mercurous chloride, potassium picrate, nitrobenzene, methylene blue, cerulein, gallocyanin, crystal violet, malachite green, and methyl orange were not reduced. Keeping the serum from contact with air by a thick layer of neutral cottonseed oil, petroleum ether, or paraffin, did not affect the result. The only dyes it reduced were indigo carmine, phenolphthalein, and alizarin disodium sulphonate. Indigo carmine was altered only after several days. Phenolphthalein was but slightly affected. Alizarin disodium sulphonate was easily decolorized. The latter was somewhat reduced even by coagulated serum. Potassium ferricyanide was rapidly reduced. This effect was weakened by boiling the serum but could not be prevented completely. Egg albumin, in solutions of about the same alkalinity as the serum, was found also to reduce potassium ferricyanide, though not as powerfully as *Limulus* serum did. If the weakly alkaline solution was first boiled it reduced ferricyanide very powerfully. As hydrogen sulphide itself reduces ferricyanide powerfully, this phenomenon was possibly due to the thio group of the haemocyanin molecule.

Nitrate was not reduced to nitrite by *Limulus* serum. The method used was that recommended by Heffter.[6] As the blood

of moribund animals and blood that is not absolutely fresh may give nitrite reactions, the serum for these experiments was drawn from a freshly caught, healthy animal, and tested as soon as possible after the blood was drawn. It is not necessary to assume that the presence of nitrite in the blood of moribund animals or in old blood is due to bacterial action. It may be due to the oxidation of ammonia, for Schönbein[9] has shown that when metallic copper is dissolved by ammonia in air, the ammonia is oxidized to nitrous acid. Moreover, Loew[10] extended this observation to completed solutions of cupric oxide in ammonia. The copper in the serum of *Limulus* is loosely combined with the protein, and the serum is very alkaline. Moreover, the absorption spectrum given by a weakly alkaline solution of pure haemocyanin is similar to that of an ammoniacal copper solution. No distinct absorption bands were found to exist in serum of *Limulus*, when such tests were made for me by Professor Gregory P. Baxter. It is probable, therefore, that the copper in the serum is in a state not unlike that in ammoniacal solutions.

Since Phisalix found that oxalates hasten the reduction of haemocyanin in serum it seemed of interest to learn what oxidizing action, if any, pure solutions of haemocyanin might exert on potassium oxalate. The haemocyanin was prepared from fresh serum by fractional precipitation with ammonium sulphate. The precipitated haemocyanin was dissolved in water while still moist, and in the presence of toluene freed from ammonium sulphate by dialysis. The haemocyanin concentration of the resulting solution was estimated by determining the nitrogen by the Kjeldahl method. The solution used contained 10.08 gm. of haemocyanin in 100 cc. 20 cc. of this solution, equivalent to 2.016 gm. of haemocyanin, 5 cc. of a solution of potassium oxalate, equivalent to 0.0241 gm. of oxalic acid, and a few drops of toluene were put in small tightly stoppered glass flasks and allowed to stand in diffused light. They were shaken several times daily to keep the solution saturated with air. Before being

[9] Schönbein, C. F., Ueber eine eigenthümliche Erzeugungsweise der salpetrigen Säure, *Monatsb. der Königlichen Preussischen Akad. d. Wissensch. z. Berlin*, 1856, 580.

[10] Loew, O., Kupferoxyd-Ammoniak als Oxydationsmittel, *Jour. f. prakt. Chem.*, 1878, xviii, 298.

set aside several of the flasks were rendered alkaline with 1 cc. $\frac{N}{10}$ KOH. At different intervals the oxalic acid was determined by acidifying with sulphuric acid, adding several volumes of alcohol, filtering, and extracting the precipitate thoroughly with alcohol. The combined alcoholic filtrates were concentrated to small bulk on the water bath. The concentrated solution was again thoroughly extracted with alcohol and the alcoholic extracts were concentrated. The concentrate was then exhausted with ether, the ether evaporated, the residue dissolved in water, filtered, and the oxalic acid precipitated as the calcium salt. This was gathered on an ashless filter, ashed, and heated to constant weight. The weights of the CaO and the conditions under which they were obtained are recorded in the following table.

TABLE II.

The Effect of Pure Haemocyanin on Potassium Oxalate.

	CaO recovered.	Time elapsed.
	gm.	*days*
0.0241 gm. potassium oxalate + haemocyanin...	0.0165	
0.0241 " " " + "	0.0101	4
0.0241 " " " solution alone.....	0.0171	7
0.0241 " " " + haemocyanin...	0.0092	7
0.0241 " " " + "	0.0176	10
0.0241 " " " + " + 1 cc. $\frac{N}{10}$ KOH	0.0098	10
0.0241 " potassium oxalate + haemocyanin + 1 cc. $\frac{N}{10}$ KOH	0.0176	10

The results from these preliminary experiments are practically negative. The variations seem to be within the range of experimental error. To learn whether a substance more easily oxidizable than oxalic acid is attacked, some of the haemocyanin solution was rendered 1 per cent alkaline with potassium hydroxide and sufficient pure anhydrous glycerol added to make the concentration of glycerol 5 per cent. At a temperature of 37°C. this was tested for oxygen absorption according to the method of Bunzel.[11] No absorption of oxygen was observed.

[11] Bunzel, H. H., The Measurement of the Oxidase Content of Plant Juices, *U. S. Dept. of Agriculture, Bureau of Plant Industry, Bull. 238*, 1912.

Freshly drawn blood frequently requires 24 hours or more to decolorize. This is particularly true if the animal has been kept in the air before bleeding until the surface of the body where the blood is drawn becomes dry. Blood that has once become decolorized, if subsequently oxygenated by shaking in air, thereafter decolorizes rapidly. The older the blood the more rapidly the blue color fades. This fact renders it altogether unlikely that the fading is due to the action of a reducing enzyme, to the presence of an auto-oxidizable substance in the blood, or to the consumption of oxygen by blood cells or by pieces of protoplasm which may have escaped entanglement in the agglutination which forms the clot. An enzyme would probably be more active in fresh blood. An auto-oxidizable substance would gradually be oxidized so that the reducing action would become progressively weaker. Cells and protoplasm would be most active in fresh blood. The simplest explanation of the reduction of the oxyhaemocyanin is that it is largely due to the development of microorganisms, which accords with the observations of Kobert.[7] This would explain the inhibiting action of heat and of antiseptics. It would also explain why old blood decolorizes more rapidly. The only argument against this view is that dialysis retards the reduction. The probable explanation of this phenomenon is that the removal of salt and other nutritive material, as well as the diminution of alkalinity, creates a condition less favorable to the growth of microorganisms, particularly that of marine organisms. As a result of the diminution of alkalinity the copper is probably more easily dissociated, for it has been shown that long continued dialysis, as well as very weak acid, may remove copper.[7] As copper is highly toxic to many microorganisms, even in great dilutions, as shown by Kellerman and Beckwith,[12] this may account for the delayed reduction. Certainly, pure haemocyanin solutions do not putrefy easily, even serum being more permanent than ordinary protein solutions.

On the whole, the conclusion seems justified that the color reduction phenomena are, *extra corpore*, due to the action of microorganisms. It cannot, however, be denied that the presence of

[12] Kellerman, K. F., and Beckwith, T. D., The Effect of Copper upon Water Bacteria, *U. S. Dept. of Agriculture, Bureau of Plant Industry, Bull. 100*, pt. vii, 1907.

reducing substances in the serum may play a part, for these may be present, as shown by the following experiments. When the blood of a well nourished, recently captured animal was treated with several volumes of alcohol, and the alcohol drained off and concentrated, the residue from the alcoholic filtrate reduced Fehling's solution. Concerning the exact cause of this reduction no observations are recorded. When the blood of animals in an advanced stage of starvation was treated in this way no reduction was observed.[13] The hypothesis advanced in this paper to account for the reduction on standing of oxyhaemocyanin in serum of *Limulus* is in accord with the observations of Alsberg and Clark[14] on the oxygen capacity of the haemocyanin of *Limulus*, that the oxyhaemocyanin is not easily dissociated and does not give off oxygen under diminished pressure.

It would appear, therefore, that the reduction on standing of oxyhaemocyanin observed in the serum of *Limulus* is probably due neither to enzymes nor to the presence of auto-oxidizable substances, but to the action of microorganisms. This was shown by determining the behavior of a large number of readily auto-oxidizable substances in presence of the serum.

[13] Alsberg, C. L., and Clark, E. D., The Blood Clot of *Limulus polyphemus*, *Jour. Biol. Chem.*, 1908–09, v, 323.

[14] Alsberg, C. L., and Clark, W. M., The Solubility of Oxygen in the Serum of *Limulus polyphemus* L. and in Solutions of Pure *Limulus* Haemocyanin, *Jour. Biol. Chem.*, 1914, xix, 503.

GASTRO-INTESTINAL STUDIES.

XI. STUDIES ON THE RELATIVE DIGESTIBILITY AND UTILIZATION BY THE HUMAN BODY OF LARD AND HYDROGENATED VEGETABLE OIL.

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A great deal of work has been done on the digestion and absorption of fats in general, and as there has been more or less discussion concerning the availability of hydrogenated vegetable oils, it was thought advisable to compare the respective behaviors of lard and a hydrogenated vegetable oil upon ingestion by the human subject.

Munk[1] and Arnschink[2] were among the earlier workers who showed that the completeness with which fats are absorbed depends on the character of the fats ingested. Their work indicates that fats of high melting point are absorbed to a smaller degree than fats of low melting point. The kind of fat ingested has also an influence on the rapidity of absorption, as shown by Munk and Rosenstein. High melting mutton fat, for example, is absorbed more slowly than lipanin, which is liquid at ordinary temperature.

Bloor[3] in some recent work obtained data of slightly different character, which indicate rather strongly that the human and animal intestine, in general, is capable of transforming the greatly varying fats of the ordinary mixed diets into fat which is uniform and more or less characteristic of the species. Bloor[4] emphasizes also the probability that saponification is a necessary preliminary to fat absorption.

There has been but little work done in regard to the relative degree of utilization of lard[5] and hydrogenated vegetable oils.

[1] Munk, I., *Arch. f. path. Anat.*, 1880, lxxx, 10; 1884, xcv, 407; 1890, cxxii, 302. Munk, I., and Rosenstein, A., *ibid.*, 1891, cxxiii, 230, 484.

[2] Arnschink, L., *Ztschr. f. Biol.*, 1890, xxvi, 434.

[3] Bloor, W. R., *Jour. Biol. Chem.*, 1914, xvi, 517.

[4] Bloor, W. R., *Jour. Biol. Chem.*, 1913, xv, 105.

[5] Langworthy, C. F., and Holmes, A. D., *U. S. Dept. of Agriculture Bulletin*, 1915.

Because of the importance to which these substances have risen on account of their increasing use for human consumption, the following experiment was conducted. The subjects of the experiment were two members of the staff of the Department of Physiological Chemistry of the Jefferson Medical College and were, as far as known, normal individuals. Their weights at the start of the experiment were 113.5 pounds (Subject S) and 132.3 pounds (Subject M).

The experiment was conducted in two periods of 8 days each, separated by an interval of 3 days. The diets of the two periods were essentially the same, with the exception that in the first period the fat of the diet was made up principally of lard, while in the second period lard was replaced by the hydrogenated vegetable oil. During the 3 day interval between the periods an ordinary mixed diet was eaten by both subjects, in order that the second period might be started under conditions as nearly like those of the beginning of the first period as possible. The diets consisted of the following substances: shredded wheat, meat, biscuits, potato chips, milk (small quantity with cereal), apple, sugar, jelly, agar-agar, and water. The diets were so arranged that the amount of fat, other than lard or hydrogenated vegetable oil, was reduced to the minimum. The amount of total fat ingested during each period by each subject is given in Table III.

The hydrogenated oil in question was made from cottonseed oil. Hydrogen was added in the presence of a catalytic agent consisting of a treated mixture of nickel salts and kieselguhr. After hydrogen had been added to the desired point, the catalytic agent was filtered out, and the hydrogenated fat thoroughly deodorized.

The daily feces of each subject were collected, thoroughly mixed, weighed, and sampled for analysis.

Analytical Procedures.

Determinations of total fat, fatty acid, and neutral fat in the daily feces were made by the Saxon method[6] which proved to be

[6] Saxon, G. J., *Jour. Biol. Chem.*, 1914, xvii, 99. For discussion see Smith, C. A., Miller, R. J., and Hawk, P. B., *ibid.*, 1915, xxi, 395.

TABLE I.

Fat Eliminated in Feces during Both Periods.

Subject S.

Stool.	Weight of stool.		Total fat.		Fatty acid.		Neutral fat.	
	Period.		Period.		Period.		Period.	
	I.	II.	I.	II.	I.	II.	I.	II.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	42.0	254.1	1.39	3.64	0.28	1.42	1.11	2.22
2	90.6	231.7	3.47	4.67	1.33	2.04	2.14	2.63
3	139.8	107.7	4.18	3.32	1.83	1.19	2.35	2.13
4	245.3	377.5	6.00	10.15	2.84	4.19	3.16	5.96
5	189.5	79.0	3.77	3.22	1.59	1.42	2.18	1.80
6	142.2	254.1	3.12	8.37	1.51	3.43	1.61	4.94
7	179.3	114.0	4.54	3.30	1.72	1.34	2.82	1.96
8	103.4	178.2	2.41	5.16	1.00	1.82	1.41	3.34
9	99.3	87.8	2.92	3.41	0.33	0.49	2.59	2.92
10	34.8		1.81		0.50		1.31	
Totals			33.61	45.24	12.93	17.34	20.68	27.90
Daily averages			4.20	5.65	1.62	2.17	2.58	3.48

Period I, lard; Period II, hydrogenated vegetable oil.

TABLE II.

Fat Eliminated in Feces during Both Periods.

Subject M.

Stool.	Weight of stool.		Total fat.		Fatty acid.		Neutral fat.	
	Period.		Period.		Period.		Period.	
	I.	II.	I.	II.	I.	II.	I.	II.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
1	172.2	92.9	4.00	3.95	1.39	1.96	2.61	1.99
2	86.0	179.3	2.31	7.81	0.95	1.89	1.36	5.92
3	191.0	280.5	3.82	9.05	1.99	4.49	1.83	4.56
4	111.8	189.5	3.44	7.03	1.64	3.45	1.80	3.58
5	220.9	253.2	5.58	6.76	3.08	3.79	2.50	3.06
6	190.7	421.7	6.43	8.52	1.58	4.47	4.85	4.05
7	448.2	387.0	8.15	6.65	3.58	3.60	4.57	3.05
8	346.9	425.5	7.95	6.51	2.36	3.57	5.59	2.94
9	95.0		3.40		1.36		2.04	
Totals			45.08	56.28	17.93	27.13	27.15	29.15
Daily averages			5.63	7.03	2.24	3.39	3.39	3.64

Period I, lard; Period II, hydrogenated vegetable oil.

TABLE III.

Digestion and Utilization of Fat for Both Periods.

	Subject S.				Subject M.			
	Period.				Period.			
	I.		II.		I.		II.	
	Total.	Daily average.	Total.	Daily average.	Total.	Daily average.	Total.	Daily ave[illegible]
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm* [illegible] *gm*
Total fat ingested...	736.1	92.0	753.1	94.0	755.3	94.4	772.3	96[illegible]6..
Fatty acid eliminated in feces.....	12.9	1.6	17.3	2.2	17.9	2.2	27.1	3[illegible]3.4
Neutral fat eliminated in feces.....	20.7	2.6	27.9	3.5	27.2	3.4	29.2	3[illegible]3.6
Amount of fat digested (split).....	715.4	89.3	725.2	90.7	728.1	91.0	743.1	9[illegible]2.9
Percentage of fat digested.....	**97.1**		**96.3**		**96.4**		**96.3**	
Total fat eliminated in feces.....	33.6	4.2	45.2	5.7	45.1	5.6	56.3	[illegible].0
Amount of fat utilized.....	702.5	87.8	707.9	88.5	710.2	88.8	716.0	8[illegible].5
Percentage of fat utilized.....	**95.4**		**94.0**		**94.0**		**92.7**	

TABLE IV.

Comparative Digestion and Utilization of Lard and Hydrogenated Vegetable Oil.

Subject.	Digestion.		Difference.	Utilization.		Difference.
	Lard.	Hydrogenated vegetable oil.		Lard.	Hydrogenated vegetable oil.	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
S.....	97.1	96.3	0.8	95.4	94.0	1.4
M.....	96.4	96.3	0.1	94.0	92.7	1.3
Averages....	96.75	96.3	0.45	94.7	93.35	1.35

speedy, convenient, and accurate. The amounts of total fat, fatty acid, and neutral fat eliminated by the two subjects during both periods are given in Tables I and II. The values for fatty acid are given, in all cases, in terms of stearic acid; the neutral fat is taken as the difference between total fat and fatty acid.

Fat was determined in the solid foods by extraction of the dried, pulverized material for 20 hours according to the usual Soxhlet procedure. The fat of the milk was determined by the Babcock method.

DISCUSSION.

In regard to the relative digestion and utilization by the human subject of lard and the hydrogenated vegetable oil used in this experiment, the experimental data, as summarized in Table IV, show practically no difference in the behavior of the two fats. The average percentage of *digestion* of lard was 96.75, and of the hydrogenated vegetable oil 96.3; while the average percentage of *utilization* of lard was 94.7, and of the hydrogenated vegetable oil 93.35. There is thus seen to have been a difference of less than 0.5 per cent (0.45) between the digestion values of lard and the hydrogenated vegetable oil, and a difference of slightly more than 1 per cent (1.35) between their respective utilization values. These differences are so small that they can be considered to be within the limit of accuracy of the experiment, and indicate rather conclusively that *as a food for man the hydrogenated vegetable oil used in this experiment is as available as lard.* There is little reason for thinking that this would not be the case. On the contrary one might readily suppose, in view of the work of Munk[1] and Arnschink[2] that the hydrogenated vegetable oil, which had a melting point of about 36°C., would have been better utilized than the lard which melted at about 45°C. The closeness in the utilization values of lard and the hydrogenated vegetable oil might possibly be explained in the light of the work of Bloor,[3] which showed the ability of the intestine to transform varying fats ingested into a more or less uniform fat suitable for use by the organism.

Different investigators have done work to show the amounts of fat absorbed by both man and animals. Rubner[1] has shown that the human

[1] Rubner, M., *Ztschr. f. Biol.*, 1879, xv, 159.

intestine can absorb over 300 gm. per day. Voit[8] demonstrated that o of 350 gm. of fat ingested by a dog, 346 gm. were absorbed from the int tinal canal. Cammidge[9] estimates that on the average about 95 per ce of the fat of a mixed diet is absorbed by healthy adults, and that from 1 6 gm. with an average of 3 gm. of fat appear in the stools. That the fo in which the fat is presented has an influence on the percentage of absor tion has been shown by Gaultier.[10] who found that only 82.6 per cent of gm. of fat as bacon was utilized, while when the same amount was giv as milk, 96.7 per cent was absorbed.

In our experiment the percentages of digestion and utilization of the two fats (Table IV) agree well with the findings of other investigators. The average amounts of total fat eliminated daily were, for Subject S, 4.20 gm. (Period I) and 5.65 gm. (Period II), while for Subject M they were 5.63 gm. and 7.03 gm. respectively.

Subject S gained 4 pounds during the first period and 3.3 pounds during the second period, while Subject M gained 4.7 pounds and 2.5 pounds respectively during the two periods. This gain in weight was apparently due to the large quantity of food ingested, as this amount was somewhat greater than that to which either subject had been accustomed, rather than to any particular influence of either lard or the hydrogenated vegetable oil.

In connection with this experiment, the work of Lehmann,[11] as well as that of Thoms and Müller,[12] and Süssmann,[13] is of interest. They all have shown that hydrogenated vegetable oils (peanut oil, sesame oil, and cotton-seed oil) are without injurious effects when ingested by dogs and human subjects, and that these hydrogenated fats are efficiently utilized. Some of the findings of Thoms and Müller indicate even a better utilization of the hydrogenated fats than lard. As concerns the content of nickel in the hydrogenated fats, Lehmann found from 0.1 mg. (minimum) to 6.0 mg. (maximum) of nickel per kilo of the hydrogenated fat, and Thoms and Müller[11] obtained values ranging from 0.83 mg. to 3.8 mg. per kilo of fat. Previous investigations by Lehmann[10] have shown that the ingestion of at least 100 mg. of nickel per day is without injurious effects to man.

---

[8] von Pettenkofer, M., and Voit, C., *Ztschr. f. Biol.*, 1873, ix, 1.

[9] Cammidge, P. J., The Faeces of Adults and Children, Bristol, 1914, 262.

[10] Gaultier, quoted by Cammidge, *loc cit.*

[11] Lehmann, K. B., *Chem. Ztg.*, 1914, xxxviii, 798.

[12] Thoms, H., and Müller, F., *Arch. f. Hyg.*, 1915, lxxxiv, 54.

[13] Süssmann, P. O., *Arch. f. Hyg.*, 1915, lxxxiv, 121.

We wish to take this opportunity of expressing our thanks to Miss Helen E. Gilson, Dietitian of the Jefferson Hospital, for her careful and accurate work in the preparation of the materials of the diets.

CONCLUSION.

The hydrogenated vegetable oil used in this experiment was as satisfactorily digested and utilized by normal men as was lard.

THE CHLORIDES IN DIABETES AFTER PANCREATECTOMY.

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The statements in the literature regarding the chloride excretion in diabetes are conflicting. Woodyatt believes in increased chloride excretion because of the increased permeability of the kidneys. But phlorhizin which increases the permeability of the kidneys to sugar has no effect on the chloride excretion.[1] This indicates that in the kidney we are dealing with specific and not with general permeability. One may also reason, in accordance with the results found by Rulon and Hawk[2] in the normal individual, that the increased water ingestion implied by the polydipsia of diabetes would tend to increase the elimination of chlorides. Von Noorden[3] states that the excretion of chlorides is seldom below normal, often running high with the nitrogen, but he admits that the difference may merely be one of food intake. Külz,[4] Tenbaum,[5] and Moraczewski[6] give data indicating that the excretion is largely a matter of individual variation. Allen[7] asserts that no difference is due to diabetes *per se*, but that the increased excretion is merely due to the increased quantity of food eaten, especially protein. Steinberg,[8] working in this laboratory on the gastric juice as collected from Pawlow pouches, found that the chloride content remained fairly constant, and was not affected by the diabetes after pancreatectomy.

On the other hand, a belief in a chloride derangement in diabetes is not wanting. Martin Fischer[9] has recently extended his colloid-chemical the-

[1] Sollmann, T., *Am. Jour. Physiol.*, 1902-03, viii, 155; 1903, ix, 425, 434, 454. Sollmann, T., and Hatcher, R. A., *ibid.*, 1905, xiii, 241, 291.

[2] Rulon, S. A., Jr., and Hawk, P. B., *Arch. Int. Med.*, 1911, vii, 536.

[3] Von Noorden, C., Metabolism and Practical Medicine, Chicago, 1907, iii, 613.

[4] Külz, E., Beiträge zur Pathologie und Therapie des Diabetes mellitus, Marburg, 1874, 130.

[5] Tenbaum, E., *Ztschr. f. Biol.*, 1896, xxxiii, 379.

[6] Moraczewski, W. v., *Ztschr. f. klin. Med.*, 1898, xxxiv, 59.

[7] Allen, F. M., Studies concerning Glycosuria and Diabetes, Boston, 1913, 145.

[8] Steinberg, personal communication.

[9] Fischer, M. H., *Jour. Am. Med. Assn.*, 1915, lxiv, 325.

ory of water absorption by protoplasm to the absorption of sodium chloride. He found that gelatin or fibrin previously placed in acid absorbed more sodium chloride than when not so sensitized. On this basis he claims that chloride retention follows acid accumulation in the body, and cites diabetes among his typical examples. McLean[10] in working out the laws of Ambard on the excretion of sodium chloride, found that in diabetic patients sodium chloride was excreted in about half the cases at a diminished threshold, this condition being the more constant in the more serious cases. The data from the patients examined show, however, a daily chloride excretion no different from the normal. Rabens[11] incidentally found in examining the urine of dogs before and after complete pancreatectomy that the chloride excretion in the diabetic condition fell to about one-seventh of that found when normal. He cites as typical that a dog which when normal excreted an average of 0.387 gm. chloride, after pancreatectomy eliminated an average of 0.054 gm. These data are especially interesting, since the dogs were under a careful régime, receiving a constant diet of beef heart, and a constant amount of water given regularly by stomach tube. The report of Verhaegen[12] also deserves mention. He found an absence of hydrochloric acid in the gastric contents of three human diabetics examined. Other clinicians, on the contrary, talk of hyperacidity.[13] Of further interest are the facts that intravenous injection of sodium chloride produces glycosuria;[14] and that intraperitoneal injection of large doses of dextrose increases the excretion of chlorides.[15]

EXPERIMENTAL PROCEDURE.

Operation.—The diabetes was produced in dogs either by total pancreatectomy in one step, or by removal of the pancreas in two stages after the method of Hédon.[16] Where the pancreas was removed at one operation, a small portion (less than one-tenth of the whole) was left in communication with the main duct. With this procedure a very satisfactory diabetes is produced (Allen). Some of the postoperative shock may be thus lessened, the subsequent life of the animal is probably lengthened, and for a time better digestion secured. At the first stage of the operations done according to the method of Hédon, all the pancreas was removed except the tail, which was displaced under the skin, retaining its blood supply. A drainage space, provided for the displaced pancreatic tissue, is carefully kept aseptic for the first few days after the operation. Should infection occur,

[10] McLean, F. C., *Am. Jour. Physiol.*, 1914-15, xxxvi, 357.
[11] Rabens, I. A., *Am. Jour. Physiol.*, 1914-15, xxxvi, 294.
[12] Verhaegen, A., *La cellule*, 1898, xiv, 36.
[13] Beveridge, J. W., *Am. Med.*, 1914, ix, 255.
[14] Fischer, M. H., *Arch. f. d. ges. Physiol.*, 1905, cix, 1.
[15] Nobécourt, P., and Bigart, *Compt. rend. Soc. de biol.*, 1902, liv, 1403
[16] Hédon, E., *Arch. internat. de physiol.*, 1910-11, x, 350.

ıe region about the transplanted pancreas shows a peculiar digestion with characteristic odor, and the animal dies in 36 to 72 hours from what Kirk[17] fers to as "acute pancreatic death." At the second stage of the operaon, the pancreatic transplant was removed under local anesthesia. Intration with 5 cc. of 1 per cent cocaine has been found satisfactory.

Diet of Dogs.—The dogs were fed on a standardized diet throughout ıe experiment, composed of two parts of beef heart, trimmed of fat and rerlying connective tissue, and one part (by weight) of stale bread, thorıghly dried in the room 2 or 3 days before use. The meat and bread were parately ground, mixed in the proper proportions, and repassed through ıe grinder. The mass, thus prepared, was autoclaved in Mason jars at) pounds' pressure for 30 minutes, and stored in the refrigerator until ıed. The diet was found to keep well when thus handled. After careful eighing, this food was moistened with warm water and served to the dogs, ıd was usually eaten voraciously. Generally about 30 to 40 gm. of food ere given per kilo of body weight, an amount found easily sufficient to ıep the dogs in good condition.

Collection of Urine, Feces, and Blood.—The urine was collected in bottles ·aining the metabolism cages. These bottles had been previously washed thymol, and were removed every 24 hours. The urine was examined ch day for its chlorine, ammonia, and sugar content.

The feces were collected in twenty-four samples, wrapped in paper, belled, and placed in a wire cage to dry. They were not examined until me time after the work on the dogs had been finished, when they were und perfectly dry, and in good condition.

The blood was obtained from the saphenous vein, the external jugular, by cutting the tail. In diabetic dogs, where the blood pressure was too w for these methods, the blood was sometimes drawn directly from the ıart. For the determinations, care was taken to secure as clear serum possible.

Chemical Methods.

Urine.—Chlorine was determined by the Volhard-Harvey titration ethod, ammonia by the Folin aeration method, and sugar by Benedict's ıantitative one-solution method.

Blood.—Chlorine percentage was determined by the method recently ıblished by McLean and Van Slyke.[18]

Feces.—The chloride content was determined along the lines used for ıimal products by the United States Bureau of Chemistry.[19] 10 gm. of ılverized dry feces were thoroughly impregnated with 30 cc. of 5 per ınt Na_2CO_3 in the nickel crucible, dried on a water bath, and ignited thor-

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[17] Kirk, E. G., *Arch. Int. Med.*, 1915, xv, 39.

[18] McLean, F. C., and Van Slyke, D. D., *Jour. Biol. Chem.*, 1915, xxi, 361.

[19] Official and Provisional Methods of Analysis, *U. S. Dept. of Agriculre, Bureau of Chemistry, Bull. 107*, 1912.

oughly for a long time at a low temperature; the ash was extracted with water and nitric acid, and the chlorine content determined by the Volhard-Harvey method. The use of nitric acid avoids a heavy precipitate of phosphate on the subsequent addition of $AgNO_3$ in the chloride determination.

RESULTS.

The determinations in the tables represent in each case a period of consecutive days, and it is the average of each period that is significant. The consideration of the data in such periods insures just comparisons. The daily variations are due to the fact that the 24 hour collections of urine do not represent in each case a 24 hour kidney excretion, as the contents of the bladder would not be the same at the same hour each day. In a change of diet, 3 days were allowed for complete transition before the data were considered; after an operation, a week to 10 days, so that the data submitted in diabetes may be considered uncomplicated by the effects of the anesthesia or convalescence.

No difference can be found to exist between the normal and diabetic dog in regard to the chloride elimination (Table I). Whereas 0.6256 gm. was the average of the chloride excretion in the normal condition of Dog A, 0.6151 gm. was the average in the diabetic condition. No chloride retention occurs in the diabetic dog even after 5 gm. of NaCl are added to the diet, the added chloride being excreted quantitatively. With the withdrawal of the additional NaCl, the chloride excretion returns to its former level. Aggravation of the diabetic condition by the feeding of sugar produces no change.

Table II presents data from another dog with the same negative results. A study of the feces and blood showed likewise no change between the diabetic and normal condition.

It was considered interesting to see whether the onset of diabetes produced any change in the chloride metabolism. The data were taken from a dog, prepared according to the method of Hédon, and give the daily chloride excretion before and after removal of the graft (Table III). The results were again negative.

Rabens thought that the reason he obtained a diminished excretion in the urine of diabetic dogs was that, because of the polyphagia and increased hunger, more hydrochloric acid was formed,

TABLE I.

The Chloride Excretion in Diabetes.

(Dog A, Female, 6 Kg. Fed 200 Gm. Standardized Food Daily.)

Normal.			Diabetic.																	
Ordinary diet.			Ordinary diet.					5 gm. NaCl added to diet.					Return to former diet.					10-20 gm. glucose added.				
Urine.	Cl	NH_3	Urine.	Cl	NH_3	Sugar.		Urine.	Cl	NH_3	Sugar.		Urine.	Cl	NH.	Sugar.		Urine.	Cl	NH_3	Sugar.	
cc.	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*gm.*	*cc.*	*g n.*	*gm.*	*per cent*	*gm.*	*cc.*	*gm.*	*gm.*	*per cent*	*gm.*
140	0.7390		260	0.6890	0.2267	9.3	24.2	797	3.9691				557	0.6795	0.4733	9.8	54.7	615	0.6458	0.3278	8.1	49.9
160	0.5296		369	0.5830	0.3052	7.9	29.2	483	3.9222				510	0.5712	0.4437	9.7	49.5	470	0.3760	0.4686	9.8	46.2
338	0.9261	0.1318	399	0.8698	0.2673	7.8	30.9	782	3.8709	0 4254	8.3	65.1	479	0.5413	0.3908	9.0	42.9	636	1.3483	0.3962	7.0	44.3
176	0.5403		249	0.2615		9.6	23.9	835	4.4420		6.3	52.2	567	0 9242	0.4179	8.9	50.2	538	0.1775	0.4756	7.0	37.4
190	0.6061		550	0.9350	0.3179	7.1	38.9	694	3.6227	0.4407	7.3	50.6	423	0.2918		9.7	44.1	581	0.8424	0.4912	5.3	30.6
171	0.6378		470	0.3760		8.2	38 6											350	0.1995	0.2975	6.3	18.9
230	0.5704		325	0.5915		8.1	26.4											615	0.7318	0.3751	7.5	46.3
209	0.4556	0.0878																				
Average..	0.6256			0.6151					3.7254*					0 6016					0 6173			

*5 gm. NaCl = 3.0325 gm. Cl
Food itself = 0.6151 " "
Therefore, theoretical excretion = *3.6476* " "

TABLE II.

Further Data on the Chloride Metabolism in Diabetes.

(Dog B, Female, 11.5 Kg. Fed 325 Gm. Standardized Food Daily.)

Normal.					Diabetic.										
Ordinary diet.					Ordinary diet.						5 gm. Na Cl added.				
Urine.		Feces.		Blood serum.	Urine.			Feces.		Blood serum.	Urine.			Feces.	
	Cl		Cl	Cl		Cl	Sugar.		Cl	Cl		Cl	Sugar.		Cl
cc.	*gm.*	*gm.*	*gm.*	*per cent*	*cc.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*cc.*	*gm.*	*per cent*	*gm.*	*gm.*
5.32	1.056				602	1.2401	6.0	62.7	0.084		810	3.2157	6.2	52.0	0.035
4.54	1.151				618	0.7663	6.0	64.0	0.068	0.406	863	3.1327	6.6	45.0	0.024
7.90	1.236	35.0	0.095	0.401	772	0.8492	5.0	85.5	0.065		982	3.7414	8.0	25.0	0.011
5.85	1.083				805	1.2478	6.2	50.3	0.035	0.403	840	3.6792	6.7	43.0	0.039
5.20	1.019			0.407	730	1.0439	8.6	69.0	0.046		770	3.0569	6.9		
5.11	0.915	25.7	0.026		700	0.8750	9.0	44.0	0.031	0.404	750	3.3300	7.0		
4.00	1.150				632	1.0744	6.7	107.0	0.087						
4.90	0.950			0.405	834	1.5679	5.9								
5.23	1.100	23.2	0.037												
Average....	1.0735					1.0831						3.3593			

and this passed out through the feces. Such an hypothesis is entirely untenable, since even with an added ingestion of 5 and 10 gm. of NaCl, the chloride content of the feces remained at an unchanged low value (Table IV).

TABLE III.

The Chloride Relations at the Onset of Diabetes.

(Dog C, Female, 5 Kg., with Pancreatic Graft. Fed 180 Gm. Standardized Food, with 5 Gm. NaCl Daily.)

Condition.	Urine.	Cl	NH_3	Sugar.
	cc.	*gm.*	*gm.*	
Before removal of graft.	382	3.201	0.1730	
	350	2.954	0.1271	
	250	2.615	0.2237	
	290	3.199	0.1972	
	280	3.181	0.2540	
	Average....	3.050		
Graft removed in afternoon of previous day.	360	3.078	0.2736	2.84 per cent 10.2 gm.

TABLE IV.

The Chloride Excretion of the Feces in Diabetes.

(Dog B.)

No NaCl added to diet.			5 gm. NaCl added to diet.			10 gm. NaCl added to diet.		
Feces.	Cl		Feces.	Cl		Feces.	Cl	
gm.	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*
62.7	0.134	0.084	52.0	0.068	0.035	50.0	0.177	0.088
64.0	0.107	0.068	45.0	0.053	0.024	28.8	0.174	0.050
85.5	0.076	0.065	25.0	0.025	0.011	36.9	0.129	0.047
50.2	0.070	0.035	43.0	0.091	0.039	50.2	0.119	0.061
69.0	0.070	0.046				86.0	0.091	0.078
44.0	0.080	0.031						
102.0	0.080	0.087						

DISCUSSION.

In view of the negative effect of diabetes on chloride metabolism, how are we to explain Rabens' results as to chloride retention? Among the various possibilities one might suggest that the dogs he used were afflicted with some form of pneumonia; or that his results are due to the giving of a constant quantity of water by stomach tube throughout the experiment. This artificial check to the polyuria of diabetes may have had, by some means or other, some effect on the chloride excretion.

Not only is the chloride excretion independent of diabetes and the degree of glycosuria, but it likewise seems to bear no relation to the ammonia excretion. If the increased ammonia excretion in the diabetes after pancreatectomy of dogs be considered an index of acidosis, these negative findings may be taken as failing to confirm Fischer's theory of chloride retention.

That ingestion of large amounts of NaCl does not change the amount of chloride excreted in the feces indicates that in diabetes after pancreatectomy there is no change in the chloride absorptive power of the intestine.

CONCLUSIONS.

1. The chloride metabolism of dogs in diabetes after pancreatectomy does not differ from that of dogs in normal condition.

2. Likewise no change occurs, in diabetes after pancreatectomy, in the permeability of the intestines as regards the absorption of chloride.

I am indebted to Dr. Carlson, under whose direction the work was undertaken, for suggestion, aid, and inspiration at every stage of the work.

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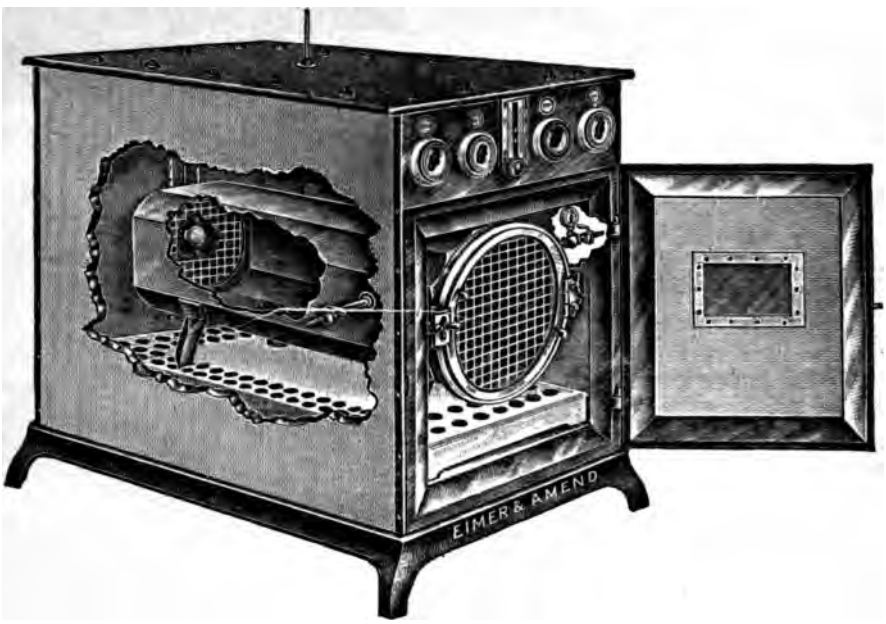

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